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<b>(54) Title:</b> COMPOSITION OF MATTER AND METHOD OF IMMUNIZING AGAINST VIRAL CAUSATIVE AGENTS OF AIDS AND ARC		
<b>(57) Abstract</b>  Composition and method for induction of a neutralizing antibody against the viral causative agents of AIDS and ARC. The composition is a conjugate of a polyamide resin and a synthetic peptide, the amino acid sequence of the synthetic peptide being sufficiently homologous to the amino acid sequence of the gp 41 and gp 120 subunits of the gp 160 envelope glycoprotein of HTLV-III, LAV or ARV to produce an immunogenic response in an experimental animal and having a hydrophilic region therein. The composition is given with or without an adjuvant in an amount effective to induce an immunogenic response, thereby protecting the experimental animal from exposure to the HTLV-III, LAV and/or ARV causative agents of AIDS and/or ARC.		

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COMPOSITION OF MATTER AND METHOD OF IMMUNIZING AGAINST  
VIRAL CAUSATIVE AGENTS OF AIDS AND ARC

STATEMENT OF GOVERNMENT INTEREST

This invention was made with government support under one or more of the following NIH grants: AI-22307-01, AI- 23619-01, and AI-23472-01. The government has certain rights in this invention.

CROSS REFERENCE TO RELATED APPLICATIONS

This is a continuation-in-part of applications Serial Numbers 790,830, filed on October 24, 1985 entitled SYNTHETIC PEPTIDES AND USE FOR DIAGNOSIS AND VACCINATION FOR AIDS AND ARC and 858,216, filed on April 30, 1986 entitled POLYAMIDE RESIN AND METHOD FOR PREPARATION OF REAGENTS FOR IMMUNODIAGNOSTIC USE.

BACKGROUND OF THE INVENTION

The present invention relates to a composition and method for vaccination against acquired immunodeficiency syndrome (AIDS) and AIDS-related complex (ARC). In particular, the present invention relates to a polyamide resin-synthetic peptide conjugate which can be used in a method of immunization against the viral causative agents of AIDS and ARC and in a diagnostic assay for AIDS and ARC.

AIDS was first discovered as a severe immune deficiency which resulted in reports of opportunistic infections occurring among male homosexuals (see Gottlieb, M.S., et al., 305 N. Engl. J. Med. 1425-1431 (1981) and Masur, H., et al., 305 N. Engl. J. Med. 1431-1438 (1981)). The incidence of this new human disease, named "acquired immunodeficiency syndrome" (AIDS), is rapidly growing. Although sexual transmission appears to be the primary mode of transfer, a number of cases in which the disorder was transferred by blood transfusion have been reported (see Gottlieb, et al., supra). The etiologic agent of this disease has

been shown to be a human retrovirus, known variously as human T lymphotropic virus type III (HTLV - III), lymphadenopathy-associated virus (LAV) (Barre-Sinoussi, F., et al., 220 Science 868-871 (1983)), or AIDS-associated retrovirus (ARV).

Seroepidemiological studies have identified HTLV-III - specific antibodies in the serum of most patients with AIDS or ARC. The predominant antigens recognized by antibodies in sera obtained from AIDS patients and from hemophiliacs are associated with the envelope glycoproteins. Further, the most immunogenic proteins of the human T lymphotropic viruses, HTLV-I and HTLV-II are cell surface-expressed glycoproteins (Chen, I.S. et al., 305 Nature (London) 502 (1983)).

The envelope (env) gene product of HTLV-III is synthesized as a polyprotein precursor and is subsequently glycosylated within infected cells. That glycosylated polyprotein, with an estimated molecular weight of 160 Kd (gp 160), is processed into an amino terminus subunit gp 120 and a carboxyl transmembrane subunit, gp 41. The gp 41 subunit is one of the predominant polypeptides in purified virus preparations.

Antibodies from AIDS and ARC patients contain viral neutralizing activity; however, infection presumably occurred in those patients prior to the development of neutralizing antibody, and whether the induction of neutralizing antibody prior to infection would result in protective immunity is unknown. The general notion with retroviruses is that the antigenic determinants or epitopes associated with the induction of neutralizing antibodies are associated with the glycoprotein envelope (see Holden, H.T. and T. Taniyama, 150 J. Exp. Med. 1367 (1979) and Flyer, D.C. et al., 305 Nature (London) 815 (1983)). Until the present invention, that association had not been established for the AIDS associated

viruses. As will be described, it has now been demonstrated that the gp 41, gp 120 and gp 160 envelope glycoproteins are the most immunogenic epitopes in virus-exposed individuals.

The present invention is premised upon the assumption that the critical epitopes involved in the induction of protective virus neutralizing antibody are associated with the two viral envelope glycoprotein subunits, gp 120 and gp 41. The present invention is also based on the use of polypeptide portions of those immunogenic subunits to induce an immunogenic response to the intact causative agents of AIDS and ARC when conjugated to the solid phase resin on which that portion of the immunogenic subunit was synthesized. Those polypeptide subunits are conveniently synthesized on solid phase resins. Solid phase peptide synthesis is a valuable tool for investigating the structure and mechanism of action of proteins and peptides (proteins and peptides are collectively referred to herein as "protides"). Most such synthetic methods involve the use of a cross-linked polystyrene based resin as the solid phase to which the protide is anchored during assembly, usually through a linker molecule. Assembly is accomplished by a repetitive cycle of adding a protected amino acid to the solid phase, selectively removing (deprotecting) a protective group on that amino acid, and adding additional suitably protected amino acids (for a review, see Merrifield, R.B., "Solid-phase Peptide Synthesis", 32 Adv. Enzymology 221 (1969)).

Although cross-linked, polystyrene based resins are most commonly used as supports in solid phase protide synthesis, their relatively hydrophobic character in comparison to the polar organic media required to solubilize reactants can be problematic in protide chain assembly. Such media may freely solvate the growing

protide, yet incompletely swell the polystyrene matrix. Within the polymer lattice, impaired diffusion of reagents and steric hindrance can contribute to lowered efficiency during coupling cycles, which, on a repeated basis, lowers final yields appreciably. During the early stages of assembly, when the resin to protide mass ratio is high and the physical properties of the support dominate, this lowered efficiency is particularly acute.

Those shortcomings led to the development of a cross-linked, polydimethylacrylamide based support which is highly polar in character and is freely permeated by the requisite solvents for peptide synthesis. Atherton, E., D.L.J. Clive and R.C. Sheppard, "Polyamide Supports For Polypeptide Synthesis", 97 J. Amer. Chem. Soc. 6584 (1975); Arshady, R., E. Atherton, M.J. Gait, K. Lee and R.C. Sheppard, "Easily Prepared Polar Support For Solid Phase Peptide And Oligonucleotide Synthesis". 1979 J.C.S. Chem. Comm. 425 (1979). That polyamide resin, as the amino methyl derivative, can accommodate synthetic schemes incorporating alternate protection strategies through selection of the appropriate linker molecule, which links the C-terminal residue to the support. However, peptides synthesized on that resin must be separated from the resin after the synthesis is completed and then purified, both time-consuming steps which decrease the final yield of the protide.

A significant advantage of the composition and method of the present invention is that the protide can be used to induce an immunogenic response in an experimental animal without being separated from the resin on which it was synthesized and then purified. The resin-protide conjugate thus synthesized can be used in a number of investigative applications. Of particular interest to the present invention is the use of certain polyamide resin-peptide conjugates,

specifically, conjugates including portions of the gp 120 and gp 41 subunits of the gp 160 envelope glycoprotein, as immunogens.

It has previously been demonstrated that synthetic peptides analogous to sequences contained in viral encoded proteins have proven useful for identification of native antigen determinants associated with such proteins. Several laboratories have reported studies on the antigenic activity of various hepatitis B antigen (HBsAg) synthetic peptides. Dreesman, G.R., et al., 295 Nature 158 (1982); Lerner, R.A., et al. 78 Proc. Natl. Acad. Sci. USA 3403 (1981); Prince, A.M., et al., 79 Proc. Natl. Acad. Sci. USA 579 (1982). The induction of an antibody response to HBsAg, using such peptides, proved to be relatively weak, but could be enhanced through coupling of peptides to a carrier protein prior to immunization. Lerner, et al., supra; Sanchez, Y., et al., 18 Intervirology 209 (1982). Further, synthetic peptides corresponding in amino acid sequence to portions of the capsid proteins of tobacco mosaic virus (Anderer, F.A. and H.D. Schlumberger, 97 Biochim. Biophys. Acta 503-509 (1965); Id. Anderer, F.A. and H.D. Schlumberger, 115 Biochim. Biophys. Acta 222-224 (1966); Fearney, F.J., C.Y. Leung, J.D. Young and E. Benjamini, 243 Biochim. Biophys. Acta 509-514 (1971)), foot and mouth disease virus (Bittle, J.L., et al., 298 Nature 30-33 (1982) and Pfaff, E., et al., 1 EMBO J. 869-874 (1982)) and poliovirus (Emini, E.A., B.A. Jameson and E. Wimmer, 304 Nature 699-703 (1983)) have been used to determine neutralizing epitopes associated with the intact virion.

Because the prediction of the potential antigenic determinants of immunogenic AIDS and ARC proteins based on primary sequence analysis of those immunogens is not exact, the identification of putative epitopes through

trial and error can be laborious. A composition and method which involves the delineation of antigenic sequences native to the viral causative agents of AIDS and ARC with synthetic peptides which does not require purification of the synthetic peptide and coupling of the peptide to carrier proteins offers significant advantages.

#### SUMMARY OF THE INVENTION

An object of the present invention is, therefore, to provide a composition of matter capable of inducing an immunogenic response to the viral causative agents of AIDS and ARC comprising a polyamide resin and a synthetic peptide, the synthetic peptide comprising a chain of amino acids having a sequence homologous to a portion of the amino acid sequence of the gp 120 or gp 41 envelope glycoprotein of HTLV-III, ARV or LAV and having a hydrophilic region therein. To accomplish that object, it was first necessary to identify a number of synthetic peptide candidates capable of eliciting such a response. It is, therefore, also an object of the present invention to characterize the amino acid sequence of the most immunogenic proteins of HTLV-III (i.e., gp 120 and gp 41), and to identify the structural conformation of those proteins and the portions of the amino acid sequence of those proteins which represent the most likely binding sites for antibodies to the intact virion, and then synthesize synthetic peptides with that same sequence and structure, or with a sequence and structure which is sufficiently homologous to the portion of the sequence which represents the binding site as to also be immunogenic.

A further object of the present invention is to provide a polyamide resin, and a method of preparing that polyamide resin, upon which those synthetic



peptides are synthesized using solid phase synthetic methods to produce a conjugate which, when injected into an experimental animal, induces an immunogenic response to the viral causative agents of AIDS and ARC without separating the synthetic peptide from the resin before injection into the experimental animal.

It is another object of the present invention to provide an assay for detecting antibodies against the viral causative agents of AIDS or ARC in those individuals suspected of having AIDS or ARC comprising conjugating a polyamide resin-peptide conjugate to the ligand of a specific binding pair wherein that binding pair is comprised of the ligand and an anti-ligand having specific affinity for that ligand and the synthetic peptide is comprised of a chain of amino acids having a sequence homologous to a portion of the gp 120 or gp 41 envelope glycoproteins of HTLV-III, ARV or LAV, contacting that conjugate with sera from an animal, thereby causing any antibodies to the viral causative agents of AIDS or ARC present in that sera to bind to that conjugate, and then contacting the bound antibodies with the anti-ligand of the specific binding pair.

It is another object of the present invention to provide a method of immunizing an experimental animal against the viral causative agents of AIDS and ARC comprising synthesizing a peptide comprising a chain of amino acids having a sequence homologous to a portion of the gp 120 or gp 41 envelope glycoprotein of HTLV-III, ARV or LAV and having a hydrophilic region therein on a polyamide resin and administering an immunogenically effective amount of the polyamide resin-peptide conjugate to an experimental animal.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is an artist's rendition of the plot of the hydrophilic averages for each residue against the amino acid sequence of the gp 160 precursor glycoprotein of the gp 120 and gp 41 env glycoproteins of HTLV-III, LAV and ARV generated by a computer program utilizing the Chou-Fasman predictive scheme for secondary structure.

Figure 2 is an actual computer plot of a segment of the amino acid sequence of the plot of Fig. 1.

Figure 3 is a schematic representation of the secondary structure of the amino acid sequence of the plot of Fig. 1 showing the differences between the secondary structure of the gp 160 precursor of HTLV-III, LAV and ARV.

Figure 4 is a graph of the optical density vs. the reciprocal dilution of the antiserum obtained from rabbits immunized with the gp 120 peptide 503-532 (Peptide 6 on Table II) showing the binding of the Peptide 6 by the rabbit antibodies by enzyme linked immunosorbent assay. Fig. 4A shows binding of Peptide 6, Fig. 4B shows the binding of a control peptide (see Example 15). Data from anti-peptide antisera from rabbit 1 is represented by a (●) and data from anti-peptide antisera from rabbit 2 is represented by a (○). Data from pre-immune sera from each rabbit is represented by a (▲) and a (Δ), respectively.

Figure 5 illustrates the growth of HTLV-III virus on the A3.01 cell line as determined by assaying reverse transcriptase (RT) activity (uptake of  $^3\text{H}$ -TTP; see Example 16). Fig. 5A shows viral replication at a  $10^{-1}$  dilution, Fig. 5B was at a  $10^{-2}$  dilution, Fig. 5C at  $10^{-3}$ , and Fig. 5D at  $10^{-4}$ .

DETAILED DESCRIPTION OF THE INVENTION

As noted above, the present invention is based in part upon the assumption that it is the gp 120 and gp 41 subunits of the envelope glycoprotein which are the most immunogenic epitopes of the viral causative agents of AIDS and ARC. As will be described, the accuracy of that assumption has now been verified. It was next necessary to determine the sequence of the amino acids of the gp 120 and gp 41 subunits and to select the portions of those envelope glycoprotein subunits which represent the most likely antibody-binding sites. This selection was accomplished by means of computer modeling of the structure of the gp 120 and gp 41 subunits.

Once the most likely sites were identified, chains of amino acids were synthesized on a polyamide resin to duplicate the amino acid sequence at each of those sites. The usual method of coupling a synthetic peptide to polystyrene based resins is through a benzyl ester derivative, and separation of the peptide from the resin is usually accomplished by either acidic or basic cleavage. Benzyl esters are susceptible to several such methods of cleavage, but are also stable throughout the multiple deprotection, neutralization and coupling reactions which are characteristic of solid phase synthetic methods. Hydrazine has also been used to separate the peptide from the resin (Kessler, W. and B. Iselin, 49 Helv. Chim. Acta 1330 (1966)) as have various ammonolytic (Manning, M., 90 J.Am.Chem.Soc. 1348 (1968)) and other methods. However, those methods all require that appropriate steps be taken to avoid damage to the peptide followed by purification of the peptide from the byproducts of the syntheses, including amino acids, short peptides, decomposition products of the resin, and sometimes, peptides containing incompletely removed protecting groups. Although purification can sometimes

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be accomplished by a direct crystallization, in synthesis in which the contaminating peptides are of approximately the same size and composition as the desired product, more selective techniques must be employed. Regardless of the method of separation and purification, those requirements add time-consuming steps to the synthesis and often lower the total yield of the synthetic peptide. The polyamide resin and method of the present invention requires no such separation and purification, thereby decreasing the amount of time required to accomplish the synthesis and raising the peptide yield.

The polyamide resin of the polyamide resin-peptide conjugate of the present invention is prepared by cross-linking a commercially available dimethylacrylamide monomer in aqueous solution using a diaminoalkane, preferably a diaminoalkane having alkenoyl groups at either end of the molecule, such as N,N'-bis-alkenoyl-diaminoalkane. In a presently preferred embodiment, the cross-linker is either N,N'-bisacrylyl-1,3-diaminopropane or N,N'-bisacrylyl-1,3-diaminobutane prepared according to the method of Halpern and Sparrow (J.A. Halpern and J.T. Sparrow, "An Improved Procedure For the Synthesis of N,N'-bisacrylyldiaminoalkanes", 10 Synthetic Comm. 569 (1980)), hereby incorporated in its totality by this specific reference thereto. The use of the propane analog is preferred because it yields a polymer of larger pore size and improved swelling properties during peptide synthesis than the polymer obtained by use of the ethyl analog. However, it will be understood by those skilled in the art who have the benefit of this disclosure that the other diaminoalkanes listed in that report, N,N'-bisacrylyl-1,2-diaminoethane and N,N'-bisacrylyl-1,6-diaminohexane, as well as other

diaminoalkanes, are also appropriate for use in the preparation of the resin of the present invention.

A functional monomer is included in the cross-linked resin. The term "functional monomer" refers to those alkenyl amines which are used to anchor the C-terminal amino acid of a synthetic peptide to the resin. The functional monomer, when protected with the methylsulfonylethyloxycarbonyl (MSC) group (see Tesser, G.I. and I.C. Balvert-Geers, "The Methylsulfonylethyloxycarbonyl Group, A New And Versatile Amino Protective Function", 7 Int. J. Peptide Protein Res. 295 (1975)), is referred to as an MSC alkenyl amine. Those functional monomers are prepared by reaction of the commercially available chloride derivative with the alkenylamine, and the MSC protective group is subsequently removed with base. However, the MSC group is not required. The polyamide resin is also prepared by simply adding an excess of the allylamine, followed by filtering or other method to remove the resulting fines. The amount of functional monomer added is selected to yield a resin substitution of between about 0.1 mmol and about 0.5 mmol per gram of resin, and preferably in the range of about 0.2 mmol to about 0.4 mmol per gram of resin. The initiator can be any of the initiators known to those skilled in the art such as a persulfate or riboflavin, and is preferably ammonium persulfate.

Because the above-described substances are combined in aqueous solution, they are collectively referred to as "the aqueous phase". The next step in the preparation of the polyamide resin of the polyamide resin-synthetic peptide conjugate of the present invention is to combine the aqueous phase with an organic phase. The term "organic phase" refers to an organic solvent which, when combined with the aqueous

phase and stirred, results in a suspension from which the resin is obtained. In a presently preferred embodiment, the organic phase comprises a mixture of hexane and carbon tetrachloride.

An emulsifier is added during the stirring to allow for the formation of beads of uniform size. The emulsifier can be any detergent known to those skilled in the art, and in a presently preferred embodiment, is either sorbitan sesquioleate, sorbitan monolaurate or sorbitan monodecanoate. The amount of detergent added is adjusted to give a spherical resin of approximately uniform size. A decrease in the amount of detergent results in an emulsion which yields increased amounts of larger, amorphous material, which could contribute to a reduction to the internal growing chains of amino acids. An increase in the amount of detergent increases the amount of fine material, which is difficult to remove without the loss of significant amounts of the resin. Those fines clog the reaction vessels of the peptide synthesizer as well as the associated lines and valves.

A promoter is then added to promote the polymerization of the monomers in the suspension, resulting in the formation of beads of the polyamide resin of the present invention. A number of promoters are known to those skilled in the art, but particular success in preparing the polyamide resin has been obtained with N,N,N',N'-tetramethylethylenediamine (TEMED). The resulting beads are then filtered and washed, the MSC group (if present) is removed with base, and the beads are dried. The beads may then be sifted through a mesh sieve to insure relatively uniform size. Overall yields using the method of the present invention ranged from about 87% to about 94% from starting monomers.

The resulting aminomethyl, cross-linked polydimethylacrylamide resin, when conjugated to the synthetic peptide, provides maximum exposure of the peptide in an aqueous solution, and the resin-polymer backbone does not restrict the peptide conformationally. The exposure of the peptide is the result of the ability of the polyamide resin to swell to many times its dry bed volume when highly solvated by water.

The synthetic peptides are synthesized on the beads by coupling to a linker which is attached to the resin with an activator. The term "linker" refers to a linking group which links the carboxyl group of the first amino acid of the synthetic peptide to the polymeric resin. In the presently preferred embodiment, this linker is an oxyalkyl benzoic acid (OBA) to which an amino acid residue is coupled to serve as the first amino acid in the peptide chain. Because the OBA linker is used to attach the C-terminal amino acid to the polyamide resin, anhydrous hydrogen fluoride can be used to remove the side chain protecting groups from the peptide without significant loss of the synthesized peptide from the resin. In the below-described examples, the amino acid of choice is glycine, which is protected with the t-butyloxycarbonyl (t-BOC) protecting group, but it will be understood by those skilled in the art who have the benefit of this disclosure that the amino acid could be any amino acid, particularly, the amino acid which is the first amino acid in the peptide to be synthesized, and that other protecting groups are equally suitable. The glycine residue serves the additional function of a spacer between the peptide and the resin-polymer backbone.

The BOC-glycyl-4-(oxymethyl) benzoic acid which is the presently preferred linker was prepared by a modification of the method described by Mitchell, et al.

(Mitchell, A.R., S.B.H. Kent, M. Engelhard and R.B. Merrifield, "A New Synthetic Route to tert-butyloxycarbonylaminoacyl-4-(oxymethyl) phenylacetamidomethyl-resin, An Improved Support of Solid-phase Peptide Synthesis", 43 J. Org. Chem. 2845 (1978)), which is incorporated herein in its totality by this specific reference thereto. An important modification of the Mitchell, et al., method is the elimination of the use of dimethylformamide as a solvent. That solvent is difficult to evaporate, consequently, even though evaporation can be hastened by raising the temperature, the method is still time-consuming. The activator used to couple the linker to the polyamide resin prepared as described above is diisopropyl carbodiimide and 4-dimethylaminopyridine, but it will be understood by those skilled in the art that other activators such as dicyclohexylcarbodiimide and 4-methylpyrrolidinopyridine are equally suitable for such a purpose.

After synthesis of the peptide on the polyamide resin, the polyamide resin-peptide conjugates of the present invention are used for a number of purposes, including in vitro assays for the presence of antibodies to HTLV-III, ARV or LAV, inducing an immunogenic response to the viral causative agents of AIDS or ARC in experimental animals, or mapping antigenic determinants on the viral causative agents of AIDS or ARC. For instance, an in vitro assay is conducted by crushing a beaded polyamide resin-synthetic peptide conjugate with a mortar and pestle and absorbing the crushed conjugate onto a solid phase such as a microtiter test plate with neutral pH buffer. Serum or other body fluid suspected of containing an antibody against the viral causative agents of AIDS or ARC is then incubated with the absorbed conjugate, unbound antibodies are removed by



washing, and the bound antibodies are detected by enzyme linked immunosorbent assay, biotin-avidin amplified assay or other detection methods such as are known in the art.

The polyamide resin-synthetic peptide conjugate can also be used to map antigenic determinants on the viral causative agents of AIDS or ARC by simply removing a portion of the polyamide resin at intervals during the synthesis of the peptide, deprotecting the peptide, and testing each removed portion in serial fashion to determine that point in the synthesis at which the peptide binds to an antibody specific for the viral causative agents of AIDS and ARC. This method is made possible by the elimination of the separation and purification steps required in other synthetic methods. The conjugate can also be tested for its ability to bind antibody by crushing and absorbing to a solid support such as a microtiter test plate and assayed as described above. Separation of the peptide from the resin and purification of the peptide is not required for such an assay.

The polyamide resin-peptide conjugates are also useful as an immunogen against the viral causative agents of AIDS or ARC. The conjugate is used directly for immunization of experimental animals with or without an adjuvant. The term "experimental animal", as used herein, refers to any animal capable of an immune response. The experimental animals of primary interest are mammals, but an immunogenic response can be induced in other experimental animals such as birds using the method of the present invention. For instance, an immune response specific for the viral causative agents of AIDS and ARC, as measured by radioimmuno-precipitation, was induced by immunization of rabbits using a conjugate comprised of a synthetic peptide with

a sequence corresponding to the protein coat of the HTLV-III virus and the polyamide resin.

Those synthetic peptides which induce rabbit antibodies which bind to AIDS virus were then tested for their ability to bind human anti-HTLV-III antibody, and the rabbit anti-HTLV-III antibodies were also tested to determine whether they were capable of neutralizing the infective virus in tissue culture. Once the most immunogenic synthetic peptides which fulfill those criteria were identified, they are used for both a vaccine and as a diagnostic assay to identify individuals exposed to the viral causative agents of AIDS and ARC as well as AIDS and ARC patients.

The amino acid sequence of the gp 120 and gp 41 subunits was determined by prediction based upon the nucleotide sequence of HTLV-III and the verification of those sequences by analysis of the sequence of the NH<sub>2</sub>-terminus by Edman degradation of the proteins labeled with <sup>3</sup>[H] leucine and <sup>35</sup>[S] cystine, as well as <sup>3</sup>[H] valine.

Verification of the immunogenic nature of the gp 120 and gp 41 envelope glycoproteins (and their precursor, gp 160) was obtained by screening serum samples from AIDS and ARC patients to identify those with antibodies against HTLV-III by indirect cell membrane immunofluorescence (MIF) using the H9/HTLV-III cell line and by radioimmunoprecipitation and sodium dodecylsulfate-polyacrylamide gel electrophoresis (RIP/SDS-PAGE) with <sup>35</sup>[S] cystine-labeled H9/HTLV-III cells. Representative antibody-positive sera were also tested on glycoprotein preparations of H9/HTLV-III cells enriched through the use of a lentil lectin column. The results indicated that the highest percentage of antibody-positive sera contained antibodies which recognized gp 120 and gp 160 and that all of the samples

which contained antibodies to other epitopes also contained antibodies which recognized gp 120 and gp 160.

Selection of the most immunogenic sites on the gp 120, gp 41 and gp 160 envelope glycoproteins was accomplished by modifying a computer program based on the hydrophilicity index described by Hopp, T.P. and K.R. Woods (78 Proc. Nat'l Acad. Sci. USA 3824-3828 (1981)) to predict the location of the hydrophilic regions associated with the HTLV-III envelope gp 160 glycoprotein from HTLV-III, LAV and ARV. The amino acid sequence of those glycoproteins was also analyzed for secondary structure using the Chou-Fasman predictive scheme (Chou, P.Y. and E.D. Fasman, 13 Biochemistry 222 (1974)). The peak hydrophilic areas were compared with the predicted secondary structure, and those areas most likely to be exposed on the surface of the glycoprotein were identified. Those areas were also examined for the presence of a  $\beta$  turn because previous studies using viral envelope proteins had indicated that the hydrophilic regions exposed on the surface with predicted  $\beta$  turn secondary structure represent immunogenic surface regions on the virus (Dreesman, G.R., et al., 295 Nature (London) 158-160 (1982) (hepatitis B surface antigen); Hopp and Woods, supra (hepatitis B surface antigen); Henderson, L.E., et al., 256 J. Biol. Chem. 8400-8406 (1981) (Raucher murine leukemia virus); Gingeras, T.R., et al., 257 J. Biol. Chem. 13475-13491 (1983) (adenovirus spike protein); Watson, R.J., et al., 218 Science 381-384 (1982) (herpes simplex virus envelope glycoprotein D)).

Having identified the sequence of the gp 160 glycoprotein from HTLV-III, LAV and ARV and the regions in that sequence which are likely to be immunogenic, the next step was to synthesize a polypeptide with the same amino acid sequence (or a sequence which is similar

enough so as to be treated in the same manner by the antibody which binds with that epitope) as that region of the glycoprotein. The synthesis was carried out by the solid-phase methodology described above. A total of ten synthetic peptides were synthesized, each selected on the basis of the above-described tests for predicted immunogenicity. The amino acid sequences of each of those synthetic peptides is given in Table II.

The ten synthetic peptides are then used to induce an immune response in rabbits by injecting the rabbits with the polyamide resin-synthetic peptide conjugate. The rabbits were also injected with synthetic peptide separated from the resin on which it was synthesized and then coupled to a carrier. The antibody titer of the rabbit sera was tested by the ability of the antibody to bind with the peptide conjugated to bovine serum albumin (BSA). Those results were confirmed by conducting inhibition studies in which the inhibition of the binding of the rabbit anti-peptide to the peptide-BSA was measured.

The rabbit anti-peptide antibodies were then examined for their ability to recognize the native proteins associated with HTLV-III. An HTLV-III infected T-cell line labelled with  $^{35}$ [S]-cystine was used for immunoprecipitation to determine whether the anti-peptide sera would bind any radioactively labelled HTLV-III native proteins. Autoradiography with SDS-PAGE confirmed that the rabbit anti-peptide antibodies specifically precipitated a single protein which corresponded to the gp 160 precursor envelope glycoprotein gp 160 of HTLV-III. The precursor gp 160 product is cleaved to yield the major gp 120 envelope glycoprotein and gp 41, the transmembrane glycoprotein. The gp 41 envelope subunit does not radioactively label to the same degree with  $^{35}$ [S]-cystine as the amino end

of the precursor gp 160 glycoprotein, and was not detected by immunoprecipitation. However, when when <sup>35</sup>[S]-methionine was used as a label, the binding was detected by immunoprecipitation, a result which has been confirmed using Western transfer methods.

The anti-peptide antibodies thus generated were then tested to determine whether they were capable of neutralizing the viral causative agents of AIDS or ARC. That determination can be made in a number of ways. In one method, the polyamide resin-peptide conjugate is crushed with a mortar and pestle, and a suspension of the resin is made in buffered saline. That emulsion of peptide is absorbed to the solid phase of microtiter plates, and nonspecific sites are blocked with 10% normal goat serum. The binding of rabbit antibodies to the peptide is detected by using biotin-goat antibody to rabbit IgG and avidin conjugated horseradish peroxidase. Peroxidase activity is determined using 1,2'-azino-di(3-ethyl-benzthiazoline-sulfonic acid) and H<sub>2</sub>O<sub>2</sub> as the substrate. A resin bound peptide corresponding to a hepatitis B surface antigen sequence serves as a control. The binding of the rabbit anti-peptide is quantified spectrophotometrically at 410 nm with a plate reader.

In a second method, the neutralizing ability of the anti-peptide antibodies was tested by incubating purified virus and rabbit anti-peptide antibodies with infected T-helper cell lines, then examining the lysed cells by Western transfer and immunoprecipitation for the presence of the virus. In a third method, the neutralizing ability of the rabbit antibodies to the polyamide resin-synthetic peptide conjugate was assessed by measuring the reduction of reverse transcriptase (RT) activity. The results were verified by radioimmunoprecipitation.

The most immunogenic synthetic peptides is then used in a diagnostic assay for AIDS and ARC and as a vaccine. When used in a diagnostic assay, the preferred method involves the detection of antibody against the viral causative agent of AIDS and/or ARC. That assay is conducted, for instance, by coating an insoluble matrix such as a column of polystyrene beads or micro-well test plate with a synthetic peptide or a synthetic peptide coupled to a carrier protein (i.e., bovine serum albumin) containing the amino acid sequence associated with the epitope(s) of one of the viral causative agents of AIDS or ARC. Alternatively, the insoluble matrix is coated with a number of different synthetic peptides (a "cocktail") containing the amino acid sequence of several epitopes. Alternatively, the polyamide resin-synthetic peptide conjugate is crushed with mortar and pestle and absorbed onto a solid phase as described above.

A sample of biological fluid from the suspected patient is incubated with the synthetic peptide-coated matrix to immunocapture the predetermined antibody. The resultant matrix, separated from the uncaptured sample, is then incubated with a quantity of biotin-labeled antibody directed to the species of the predetermined antibody (e.g., anti-human antibodies would be the predetermined antibody if the body fluid is taken from a human patient) sufficient to bind a measurable number of human antibodies, if present. The resultant matrix, separated from uncaptured biotin-labeled antibody and the matrix, is then incubated with a quantity of labeled avidin, preferably avidin labeled with an enzyme such as alkaline phosphatase, sufficient to bind a measurable number of antibodies, if present. The resultant matrix is separated from uncaptured avidin and a label detected and/or preferably quantified by adding the substrate

which is specific for that enzyme to thereby determine indirectly the presence of antibody to AIDS virus in the sample. The antibody could also be labeled with an enzyme directly, in which case the matrix is incubated with an enzyme-reactive substrate, and the change in the substrate, e.g., a color change or fluorescence emission is detected. Regardless of whether the label is an antibody, an enzyme or an enzyme labeled with biotin-avidin, the binding pair formed by the antigen and antibody or the enzyme and substrate will be referred to as the "ligand" and "anti-ligand" of the specific binding pair.

A diagnostic assay can also be conducted for detection of the antigen rather than the predetermined antibody. To conduct an antigen test, the solid phase matrix is coated with antibodies to the viral causative agents of AIDS, i.e., the antibodies produced by immunization with a synthetic peptide or polyamide resin-synthetic peptide conjugate (or, preferably, several peptides) such as the peptides of the present invention. The sample of biological fluid from a patient suspected of having been infected with the AIDS virus is then added to the matrix, followed by the addition of biotin-labeled antibody, where the antibody is an antibody which binds to the AIDS virus produced in the same way as discussed above. The avidin-labeled enzyme is then added, followed by the substrate specific for the enzyme, and the color change or fluorescence emission is detected. Either of these assays is also conducted as an inhibition assay where, instead of adding biotin-labeled antibody to the AIDS virus to the bound antigen, a biotin-synthetic peptide or biotin-polyamide resin-synthetic peptide conjugate is added.

To use the polyamide resin-synthetic peptide conjugate of the present invention as a vaccine against

the viral causative agents of AIDS, approximately 100 to 1000 micrograms of synthetic peptide, or several synthetic peptides, prepared as a polyamide resin-synthetic peptide conjugate according to the teachings of the present invention, is administered to an individual with an adjuvant. The synthetic peptides were also administered, after separation from the resin on which they were synthesized, coupled to a carrier. Appropriate carriers include the toxoid components, any one of several large protein-containing substances which are foreign to the animal to be injected, any of several small peptide preparations which have demonstrated adjuvant activity and which behave as a carrier, or liposomes. The toxoid components can be tetanus toxoid or diphtheria toxoid. The phrase "large protein-containing substances which are foreign to the animal to be injected", refers to such substances as Keyhole limpet hemocyanin (KLH) or BSA. The small peptide preparations with demonstrated adjuvant activity which also act as a carrier include muramyl dipeptide, murabutidine, and the polyamino acids such as poly-L-glutamic acid or poly-L-lysine. Approximately 10 to 100 molecules of synthetic peptide are complexed to each molecule of carrier using a heterobifunctional cross-linker such as m-maleimidobenzyl-N-hydroxysuccinimide ester (MBS) (Liu, F.T., et al., 18 Biochemistry 690 (1979), Green, N. et al., 28 Cell 477 (1982)), glutaraldehyde, a carbodiimide, succinyl anhydride or N-succinimidyl-3-[2-pyridyldithio]-propionate.

Suitable adjuvants include alum (aluminum hydroxide) and any of a number of additional adjuvants such as are known to those skilled in the art. Both the polyamide resin-peptide conjugate and the carrier-synthetic peptide complex are administered in a pharmaceutically acceptable diluent such as distilled



water, phosphate buffered saline, citrate buffer or any neutral pH buffer, i.e. a buffer with a pH of between about 6 and about 8.

The polyamide resin-synthetic peptide conjugate of the present invention is also used to screen putative vaccine candidates against AIDS and/or ARC. Such screening is best conducted by coating an insoluble matrix with crushed beads of the polyamide resin-synthetic peptide conjugate as described above. The vaccine candidate is then incubated with antibodies against the peptide (with or without biotin) such as a 1:1000 dilution of IgG-rabbit anti-peptide-biotin antibody. If biotin labeled antibody is used, the avidin-enzyme conjugate is added (if no biotin is used, add biotin-labeled anti-species (such as biotin-labeled goat anti-rabbit IgG) antibody, then add avidin-enzyme), the substrate is then added and the reaction detected.

The polyamide resin-synthetic peptide conjugate of the present invention is also used to serotype viral isolates from AIDS or ARC patients. Serotyping is conducted in the same manner as described above for screening vaccine candidates, because in both cases, the anti-peptide antibody must bind with the intact AIDS viral causative agent. However, in the case of the serotyping of the viral isolate, a portion of the isolate is added, in serial fashion, to a number of bound anti-peptide antibodies, each antibody being specific for a different polyamide resin-synthetic peptide conjugate and having been bound to a separate insoluble matrix.

The present invention may be better understood by reference to the following non-limiting examples.

Example 1. Maintenance and Radioactive  
Labeling of HTLV-III Infected Cells

Two HTLV-III producing cell lines, H-9 and MOLT-3, were grown in RPMI-1640 supplemented with 20% fetal bovine serum, 2 mM glutamine, non-essential amino acids and 0.1%  $\text{NaHCO}_3$  (maintenance medium). Cell cultures were labeled by transferring cells from maintenance medium to cystine and glucose deficient medium for 1 hour before adding  $^{35}\text{S}$ -cystine (150  $\mu\text{Ci/ml}$ ) and  $^3\text{H}$ -glucosamine (20  $\mu\text{Ci/ml}$  for 24 hr). Cells were separated from tissue culture supernatants by low speed centrifugation (1,000 x g for 10 minutes).

Example 2. Verification of Immunogenicity  
of gp 120 and gp 41 Subunits of HTLV-III

Serum samples taken from subjects who came to a community health clinic in a high-risk area for AIDS and ARC and to hospitals in that area during 1983 and 1984 were screened for antibodies to HTLV-III by indirect cell membrane immunofluorescence (MIF) using the H9/HTLV-III cell line as described by Essex, et al., 320 Science 859 (1983). Briefly, this method involves separating the cells from the media as described in Example 1, above, washing between  $1 \times 10^6$  and  $2 \times 10^6$  cells twice with phosphate buffered saline (PBS), and exposing them to 40  $\mu\text{l}$  of a 1:4 dilution of previously centrifuged serum for 30 minutes at 37°C. Each preparation was then washed twice with PBS and reacted with 40  $\mu\text{l}$  of a 1:20 dilution of fluorescein conjugated  $\text{F(ab')}_2$  fragment of goat antiserum to human immunoglobulins (IgA + IgG + IgM) (Cappel, Cochranville, Pa.). The samples were again incubated at 37°C for 30 minutes, washed twice with PBS, and examined by fluorescence microscopy. If at least 50 percent (or 40 percent when indicated) of the cells showed specific fluorescence, the serum

samples were judged positive. Samples were coded and read in a double blind manner, and positive and negative human serum samples were included as a reference. The results of this screening are presented in Table I.

TABLE I

Category	Number Tested	Number (and percent) positive for	
		HTLV-III-MA <sup>1</sup>	gp120 <sup>2</sup>
AIDS	50	48 (96)	49 (98)
ARC	50	43 (86)	46 (92)
HEALTHY HOMO-SEXUAL MALES	73	34 (47)	36 (49)
HEALTHY LABORATORY WORKERS	27	0	0

<sup>1</sup> Assay for HTLV-III membrane antigens (HTLV-III-MA) conducted by MIF as described by Essex, et al., 220 Science 859 (1983).

<sup>2</sup> Assay for gp 120 envelope glycoprotein of HTLV-III conducted by RIP/SDS-PAGE as described by Essex, et al., 220 Science 859 (1983).

All of the samples from the same 190 individuals were also tested by radioimmunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (RIP/SDS-PAGE) with <sup>35</sup>[S] cystine-labeled H9/HTLV-III and uninfected H9 cells. Briefly, that method is as follows. After disruption of the labeled cells with RIPA buffer (0.15 M NaCl, 0.05 M tris-HCl, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS), the cells were centrifuged at 100,000 x g for one hour. The lysate supernatant was cleared once with 10 µl of reference negative control serum bound to Protein A-Sepharose CL-4B (Protein A beads) before portions were

reacted with 10  $\mu$ l of the human test sera. Immunoprecipitates were eluted in a sample buffer (0.1 M Cleland's reagent, 2% SDS, 0.08 M tris-HCl, pH 6.8, 10% glycerol, and 0.2% bromophenol blue) by boiling at 100°C for two minutes. Samples were analyzed in a 12.5% acrylamide resolving gel with 3.5% stacking gel according to the discontinuous buffer system of Laemmli (227 Nature (London) 680 (1970)). Surface-labeling was carried out by lactoperoxidase-catalyzed radioiodination. The results are presented in Table II.

Representative antibody-positive sera were also tested on glycoprotein preparations of H9/HTLV-III cells enriched through the use of a lentil lectin column. HTLV-III glycoproteins were incubated with lentil lectin Sepharose 4B for four hours and then eluted with 0.2 M methyl mannoside. The resulting proteins were then immunoprecipitated with HTLV-III reference serum, and the precipitates bound to protein A-Sepharose were dissociated from antibody by boiling for two minutes in the presence of 0.1% SDS and 0.15 M sodium citrate pH 5.5. Equal portions were then incubated for three hours at 37°C in the presence or absence of 0.25  $\mu$ g of endoglycosidase H. The reaction was terminated by the addition of five volumes of cold 95% ethanol, and the proteins were precipitated overnight at -20°C. The samples were then centrifuged at 12000 x g for 15 minutes and the proteins were reconstituted with electrophoresis sample buffer, boiled for three minutes, and subjected to electrophoresis. Samples from four antibody-positive AIDS patients precipitated proteins of about 120 kD, 160 kD and 41 kD. Similar results were obtained with two antibody-positive ARC patients, and with two antibody-positive healthy homosexual males. No proteins of related sizes were detected in sera from antibody-negative healthy homosexual males or

TABLE II

	1	2	3	4	5	6	7	8	9	10
Residue Nos.	341-370	304-327	728-752	735-752	846-860	503-532	733-756	55-81	650-671	465-490
	ser	thr	leu	asp	ala	val	gly	ala	his <sup>u</sup>	gly
	arg	arg	pro	arg	ile <sup>a</sup>	ala	pro	thr	ser <sup>v</sup>	gly <sup>x</sup>
	ala	pro	ile	pro <sup>k</sup>	arg	pro	asp	thr	leu <sup>w</sup>	asn <sup>y</sup>
	lys <sup>a</sup>	asn	pro	glu <sup>k</sup>	his	thr	arg	thr	ile <sup>w</sup>	ser <sup>y</sup>
	trp	asn	arg	gly	ile	lys	pro	leu	glu	asn <sup>z</sup>
	asn <sup>b</sup>	asn	gly	ile	pro <sup>n</sup>	ala	arg <sup>q</sup>	phe	glu	asn <sup>z</sup>
	asn <sup>b</sup>	thr	pro	glu	arg	lys	gly	ser <sup>a</sup>	ser	glu <sup>aa</sup>
	thr	arg	asp	glu	arg	arg	ile	ala	gln	ser
	leu	lys	arg	glu <sup>l</sup>	ile	arg	glu	ser	asn	glu <sup>bb</sup>
	lys <sup>c</sup>	ser	pro	gly <sup>l</sup>	arg	val	glu	asp	gln	ile <sup>bb</sup>
	glu	ile <sup>h</sup>	glu <sup>k</sup>	gly	gln	val	glu <sup>l</sup>	ala <sup>t</sup>	gln	phe
	ile	arg	gly	glu	gly	gln	gly <sup>l</sup>	lys <sup>t</sup>	glu	arg
Se- quence	asp <sup>d</sup>	ile	ile	arg	leu	arg	gly	ala	lys	pro
	ser <sup>e</sup>	gln <sup>i</sup>	glu	asp	glu	glu	glu	tyr	asn	gly
	lys	arg <sup>i</sup>	glu	arg	arg	lys	arg	asp	glu	gly
	leu	gly	glu <sup>l</sup>	asp		arg	asp	thr	gln	gly
	arg	pro	gly <sup>l</sup>	arg		ala	arg <sup>r</sup>	glu	glu	asp
	glu	gly	gly	ser		val	arg <sup>r</sup>	val	leu	met
	gln	arg	glu			gly <sup>o</sup>	ser	his	leu	arg
	phe	ala	arg			ile <sup>o</sup>	ile	asn	glu	asp
	gly	phe <sup>j</sup>	arg			gly	arg	val	leu	asn
	asn	val <sup>j</sup>	arg			ala <sup>p</sup>	leu	trp	asp	trp
	asn	thr	asp			leu <sup>p</sup>	val	ala		arg
	lys	ile	arg			phe		thr		ser
	thr		ser			leu		his		glu
	ile <sup>f</sup>					gly		ala		leu
	ile <sup>f</sup>					phe		cys		
	phe <sup>g</sup>					leu				
	lys <sup>g</sup>					gly				
	gln					ala				

a	gln in ARV
b	ala in LAV
c	glu in ARV
d	val in ARV; ala in LAV
e	lys in ARV
f	val in ARV
g	asn in ARV
h	tyr in ARV
i	omitted in ARV
j	his in ARV
k	asp in ARV
l	can be asp in HTLV-III
m	leu in ARV
n	his in ARV
o	insert val between ile and gly in ARV
p	met in ARV
q	can be glu in HTLV-III
r	actual sequence of this peptide in HTLV-III, ARV and LAV includes asp between arg and arg
s	cys in HTLV-III
t	arg in ARV
u	tyr in ARV
v	thr in ARV
w	leu in ARV
x	next three residues absent in LAV; substitute thr for asn in ARV
y	asn in ARV
z	asp in ARV
aa	gly in LAV
bb	val in ARV

with sera from apparently healthy laboratory workers. None of the human serum samples tested contained antibodies to other epitopes on the HTLV-III virus without also containing readily detectible antibodies to at least gp 120 and gp 160.

Example 3. Selection of Immunogenic Sites  
on gp 120, gp 41 and gp 160 Envelope Glycoproteins

The predicted amino acid sequences of the gp 160 precursor glycoprotein from the three viral isolates HTLV-III, LAV and ARV were run through a computer program which utilizes the parameters and hydrophilic values arrived at by Hopp, T.P., and K.R. Woods (20 Mol. Immunol. 483-489 (1983)). The computer program was written in Apple BASIC. The program was written with the ability to save the amino acid sequence to disk in a format which is compatible with the Chou-Fasman predictive scheme (Chou, P.Y. and E. D. Fasman, 13 Biochemistry 222 (1974)). The hydrophilicity program calculates the hydrophilic averages over a hexapeptide length, thereby increasing the accuracy of the predictions. Since there are no hydrophilic values for Asx or Glx, the amide form of the acidic amino acid residues, those codes must be edited out before running the calculations. The plots of the hydrophilic averages per residue against the amino acid sequence number for the three AIDS viral glycoproteins are shown in Figure 1. Fig. 1 is actually an artist's rendition of the computer graphical output of the hydrophilicity plots from the three viral causative agents of AIDS/ARC which have been characterized. The highest peak (most hydrophilic) is shown in a similar area for all three sequences, with the maximum hydrophilic index occurring at residues 739, 744, and 738 for HTLV-III, LAV and ARV respectively. The second highest hydrophilic region centers around the amino acid residues 653-659 just to the amino terminal

side of peak 1. The third highest hydrophilic region was found to be in close proximity to peak 1, centered around amino acid residues 733-739 for each of the three glycoproteins.

An actual computer graph output of a segment of the HTLV-III sequence is depicted in Fig. 2. Due to the length of the entire HTLV-III sequence, only a segment is shown. A proline residue is shown graphically as a "P". Two or more aromatic amino acids in a row within the sequence are depicted as an "O". The presence of aromatic amino acids within a given sequence is indicative of regions that possess a high degree of potential for hydrogen bonding. Thus, hydrogen bonds may act to influence the overall confirmation of the protein. These data indicate that these regions are likely to be exposed on the surface of the glycoprotein.

The predicted secondary structure of the HTLV-III glycoprotein, as determined by the Chou-Fasman predictive scheme, is depicted in Fig. 3. The major differences in predicted secondary structure between HTLV-III, LAV and ARV are shown in boxed regions. These regions include residues 127-150, 127-155, and 126-148 for HTLV-III, LAV and ARV respectively, where the residue homology is only about 40%, causing changes in  $\beta$  turn potential. In addition, significant differences were noted at regions 319-330 and 398-408 of ARV, 323-333 and 401-415 of LAV, and 318-328 and 396-411 of HTLV-III. Comparison of hydrophilicity with secondary structure indicates that peak 1 contains four potential  $\beta$  turns within the region, making the region centered around amino acids 739-744 a prime candidate as a potential antigenic determinant(s). Hydrophilic peak 2 also possessed a predicted  $\beta$  turn, suggesting that this region is exposed on the surface of the envelope glycoprotein.

#### Example 4. Synthesis of Peptide 4

A synthetic peptide having the amino acid sequence shown under the "Peptide 4" heading in Table II, which corresponds to the sequence of residue numbers 735 through 752 of the gp 120 glycoprotein of the viral causative agents of AIDS and ARC, was synthesized by solid-phase methodology (Merrifield, R.B., 32 Adv. Enzymol. 221 (1969)) on a Biosearch SamII peptide synthesizer. Butyloxycarbonyl-S-4-methylbenzyl-L-cysteine coupled to polystyrene using dicyclohexylcarbodiimide with a catalytic amount of 4-N,N-dimethylaminopyridine was used as the solid-phase support for the synthesis. The four amino groups were protected with tert-butyloxycarbonyl (t-BOC) and the side chain protecting groups were as follows: benzyl ether for the hydroxyl of serine, dichlorobenzyl ether for the phenolic hydroxyl of tyrosine, and the  $\gamma$  and  $\beta$  benzyl-esters were used for the carboxyl groups on glutamic acid and aspartic acid, respectively. Trifluoroacetic acid (40% in  $\text{CH}_2\text{Cl}_2$ ) was used to remove t-BOC and the resulting salt was neutralized with N,N-diisopropylethylamine (10% in  $\text{CH}_2\text{Cl}_2$ ). Diisopropylcarbodiimide was used to couple the t-BOC amino acids. The specific steps of the synthesis are published in Sparrow, J.T., 41 J. Org. Chem. 1350 (1976), hereby incorporated in its entirety by this specific reference thereto.

The protecting groups were removed and the peptide was cleaved from the resin at 0°C with anhydrous hydrogen fluoride containing 10% anisole and 1% ethanedithiol as scavengers. The hydrogen fluoride reagent was removed under vacuum at 0°C and the peptide was then precipitated and washed with anhydrous ether. After extraction of the peptide from the resin with trifluoroacetic acid, the solvent was evaporated to 15°C



and the peptide was again precipitated with ether. The ether was decanted after centrifugation and the pellet was dissolved in 5% acetic acid with 6 M guanadine HCl.

That solution was desalted on a BioGel P2 column equilibrated in 5% acetic acid and the peptide containing fractions were pooled and lyophilized. A cysteine residue was then added to the carboxyl terminus of the peptide to provide a functional -SH group for the coupling of the peptide to carrier proteins. A glycine residue was added after the cysteine to provide a spacer amino acid between the coupled cysteine residue and the amino acid sequences analogous to gp 160. A tyrosine residue was added to the amino terminus for radioactive labelling with <sup>125</sup>Iodine to determine peptide-to-carrier protein coupling efficiency and to identify the peptide during purification by adsorbance at 278 nm.

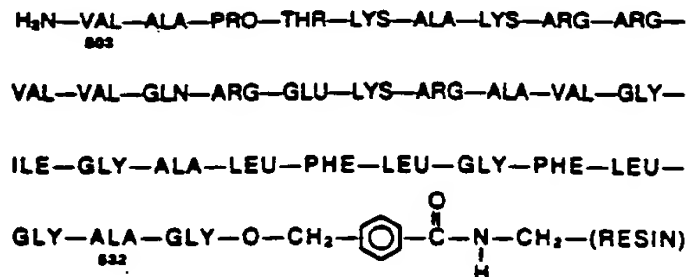
After desalting on the BioGel P2 column in acetic acid and lyophilization, the peptide was found to have the expected amino acid analysis (see Table II) and eluted as a single peak (92%) on C<sub>18</sub>-reverse-phase HPLC in a linear gradient of 0.05% trifluoacetic acid and 2-propanol.

#### Example 5.

#### Synthesis of Peptide 6 on Polyamide Resin

A synthetic peptide having the sequence shown under the Peptide 6 heading in Table II, which corresponds to the sequence of residue numbers 503-532 of the gp 120 glycoprotein of the viral causative agents of AIDS and ARC, was synthesized on a polyamide resin as follows. The numbering system used throughout this specification is taken from the numbers assigned to the amino acid residues of HTLV-III as set out in Ratner, L., et al., "Complete nucleotide sequence of the AIDS virus, HTLV-III," 313 Nature 277-284 (1985), hereby incorporated in its totality by this specific reference

thereto. The sequence of the polyamide resin-Peptide 6 conjugate was as follows:



#### A. Preparation Of Functional Monomer

Five grams of (26.8 mmol) 2-methylsulfonyl ethyloxycarbonyl chloride (MSC chloride) (K+K Labs, ICN) were dissolved in 15 ml acetonitrile and added dropwise over a 20 minute period to a stirred solution of 2.1 ml (28 mmol) redistilled allylamine (Kodak) and 4.9 ml (28 mmol) redistilled diisopropylethylamine (DIEA) in 20 ml acetonitrile. (DIEA (Aldrich) was refluxed over ninhydrin and redistilled.) The solution was stirred an additional two hours and the solvent evaporated. The residue was taken up in 250 ml ethyl acetate and allowed to stand for one-two hours. The bulk of the DIEA hydrochloride salt precipitated as needles. After filtration and evaporation, the crude material was dissolved in a minimal amount of chloroform and loaded onto a silica gel G-60 column (60 g) packed in the same solvent. Elution with chloroform yielded pure MSC-allylamine. ( $R_F$  on TLC = .64 (Solvent =  $\text{CHCl}_3$ :  $\text{CH}_3\text{OH}$ , 9:1).)

The remaining DIEA salts adsorbed to the column under these conditions. Occasionally, material migrating near the solvent front on TLC contaminated the MSC-allylamine column fractions. That material was

removed by crystallizing the MSC allylamine from methylene chloride-hexane at  $-20^{\circ}\text{C}$ . Yield was 4.8 g (86% from MSC chloride).

#### B. Preparation of Cross-linker

The cross-linker N,N'-bisacrylyl-1,3-diaminopropane was prepared according to the method set out in Helpert and Sparrow, supra. Briefly, diaminopropane (Aldrich) was dissolved in acetonitrile and added dropwise to an acrylyl chloride-acetonitrile solution at  $4^{\circ}\text{C}$ , allowed to warm to room temperature and stirred. The diaminopropane dihydrochloride was removed by filtration, washed with warm acetonitrile, and the combined filtrates were concentrated in vacuo. N,N'-bisacrylyl-1,3-diaminopropane was crystallized at  $4^{\circ}\text{C}$  overnight and the resulting plates filtered and dried in vacuo.

#### C. Preparation of Polyamide Resin

In a glass, 2-liter cylindrical, fluted polymerization vessel fitted with a nitrogen inlet and mechanically driven glass stirrer were added 490 ml hexane and 290 ml carbon tetrachloride. The solution was purged for 15 minutes with nitrogen to remove oxygen. To this solution was added an aqueous solution containing N,N'-bisacrylyl-1,3-diaminopropane (2.9 grams, 15.9 mmol) prepared as described in Example 5.B. mixed with 18.2 ml (175 mmol) of N,N-dimethylacrylamide (PolySciences). Ten g (48 mmol) MSC allylamine prepared as described in Example 5.A. and 120 ml water were added, and the solution was filtered and degassed before addition to the organic phase. The density of the resulting mixture was adjusted to obtain a uniform suspension with stirring at 400-450 RPM. Ammonium persulfate (BioRad) (0.5 g in 5 ml  $\text{H}_2\text{O}$ ) and 1 ml of either sorbitan sesquioleate or sorbitan monolaurate (Sigma) were added. A solution of 3 ml N,N,N',N'-

tetramethylethylenediamine (TEMED) (BioRad) in 2 ml H<sub>2</sub>O, pH 6.5-7.5 (conc. HCl) was then added to the suspension. The suspended emulsion was stirred for two hours under nitrogen atmosphere. The resultant beaded material was then filtered and washed sequentially with water (one liter) methanol (one liter), a mixture of dioxane:methanol:2 N NaOH (14:5:1, two liters, to remove MSC group), water (two liters), 1 N HCl (two liters), water (two liters), and then methanol (two liters). The resin was defined by suspension in methanol and decanting (3x). After swelling in methylene chloride (Baker HPLC grade), the resin was shrunk in hexane and dried in vacuo. Large amorphous material was removed by sifting the resin through an 80 mesh (180 micron) sieve.

The degree of functionalization was checked by coupling BOC-alanine to 100 mg of the resin using diisopropylcarbodiimide as activator and 4-dimethylaminopyridine (recrystallized from ethyl acetate) as catalyst. Amino acid analysis showed a substitution of 0.15 to 0.35 mmol/g resin, depending on the lot, and resins were prepared with as little as about 0.1 and as much as about 0.5 mmol/g resin depending upon the amount of allylamine added. The loaded resin gave no detectable staining with picryl-sulfonic acid, indicating the absence of unreacted free amine. When swollen in methylene chloride, the beads occupied about 2.5 times their dry bed volume. When swollen in dimethylformamide or an aqueous solution, the beads occupied approximately four and six times their dry bed volume, respectively.

#### D. Preparation of Linker

The linker BOC-glycyl-4-(oxymethyl) benzoic acid was prepared by modification of the method of Mitchell, et al., supra. Briefly, the 4-(bromomethyl) benzoic

acid phenylacylester was prepared by dissolving 10.3 ml redistilled diisopropylethylamine and 12.05 g (60.6 mmol) bromoacetophenone in 450 ml ethyl acetate. 4-(bromomethyl) benzoic acid (13.89 g, 60.6 mmol) was added in seven equal portions over a three hour period to the stirred solution at 40-50°C. Stirring was continued for two more hours at room temperature. Precipitated Et<sub>3</sub>N HBr was removed by filtration and the ethyl acetate solution was washed four times with 50 ml each of an aqueous solution of 10% citric acid, saturated sodium chloride, saturated sodium bicarbonate, and saturated sodium chloride. The organic phase was dried over anhydrous magnesium sulfate and freed of solvent by rotary evaporation under reduced pressure. The residue was crystallized from CH<sub>2</sub>Cl<sub>2</sub>-petroleum ether (1:3 v/v) to give the 4-(bromomethyl) benzoic acid phenylacylester.

The 4-(bromomethyl) benzoic acid phenylacylester was converted to BOC-glycyl-4-(oxymethyl) benzoic acid by dissolving BOC-L-glycine (25 mmol, 4.38g) in 15 ml methanol and titrating to neutrality with tetramethylammonium hydroxide (25% in methanol). Solvent was removed azeotropically with chloroform in vacuo, and the salt dissolved in 150 ml acetonitrile. To the stirred solution was added 5.8 g (17.5 mmol) of the 4-(bromomethyl) benzoic acid phenacyl ester prepared as described. After overnight mixing, the precipitated tetramethylammonium bromide was filtered and the solvent evaporated. The residue was dissolved in 400 ml ethyl acetate and the solution filtered. The organic phase was then washed successively with 10% aqueous citric acid (3 x 75 ml), 0.5 M sodium bicarbonate: 0.5 M potassium carbonate (2:1), pH 9.5 (8 x 75 ml), then water (3 x 75 ml). The solution was dried (MgSO<sub>4</sub>) and the solvent removed in vacuo. The residue was dissolved

in 200 ml of 85% acetic acid to which 23 g acid washed zinc dust was added. The mixture was stirred until the phenacyl ester was no longer visible by TLC (4 - 5 hours). The zinc was filtered and washed with 50 ml acetic acid, and the combined solutions were lyophilized. The residue was suspended in 100 ml water:300 ml ethyl acetate, and the pH adjusted to 1.5 (conc. HCl). The aqueous layer was extracted with a second portion of ethyl acetate (200 ml), and the combined extracts were washed with water (100 ml). After drying ( $\text{MgSO}_4$ ) and evaporating, the BOC-glycyl-4-(oxymethyl) benzoic acid was purified by recrystallization from methylene chloride:hexane at  $-10^\circ$ . Yield was 4.5 g (14.5 mmol, 83% from the phenacyl ester).

#### E. Coupling of Linker To Polyamide Resin

BOC-glycyl-4-(oxymethyl) benzoic acid prepared as described in Example 5.D. was coupled to the aminomethyl polyamide resin (1.2 g) prepared as described in Example 5.C. on a Biosearch Sam II automated peptide synthesizer using dicyclohexylcarbodiimide and dimethylaminopyridine as activator in a 1:1 methylene chloride:dimethylformamide solution. Both methylene chloride (Baker HPLC grade) and dimethylformamide (Baker Photrex grade) were used without further purification. Following treatment with hydrogen fluoride, 50 mg of the glycyl resin was found to contain 0.15 mmol/g by amino acid analysis. Amino acid analysis was performed using either (1) a Beckman Model 119 amino acid analyzer following either a two hour hydrolysis (12 N HCl:propionic acid, 1:1,  $135^\circ\text{C}$ ) or 24 hour hydrolysis (6 N HCl,  $110^\circ\text{C}$ ) of resin bound peptides or (2) a Beckman Model 7300 amino acid analyzer following a two hour hydrolysis (12 N HCl:propionic acid, 1:1, containing

0.05% phenol at 135°C. The results of the amino acid analysis are set out in Table III.

Peptides 1, 2, 7, and 10 were synthesized in the same manner on the resin to give the corresponding polyamide resin-peptide conjugates.

Examples 6-10. Synthesis of  
Additional Peptides

The method described in Example 4, above, was used to synthesize the Peptides 3, 5, 8 and 9 listed in Table II, each corresponding to the amino acid sequence of the residues listed.

Example 11.  
Conjugation of Synthetic Peptide to Carrier

Synthetic peptide 4 (see Table II) was conjugated via the -SH group on the cysteine residue to the amino groups on Keyhole limpet hemacyanin (KLH) (for immunization of rabbits) and bovine serum albumin (BSA) (for assaying anti-peptide activity) using a heterobifunctional cross-linker, M-maleimidobenzyl-N-hydroxysuccinimide ester (MBS). The details of this method are given at Liu, F.T., et al., 18 Biochemistry 690 (1979) and Green, N. et al., 28 Cell 477 (1982), both of which are hereby incorporated in their entirety by this specific reference thereto.

TABLE III

Residues	Before HF Treatment	After HF Treatment
Thr	.75 (1) <sup>a</sup>	.85 (1)
Glu/Gln	2.30 (2)	2.15 (2)
Pro	N.D. <sup>b</sup> (1)	1.07 (1)
Gly	4.85 (5)	5.35 (5)
Ala	4.70 (5)	5.19 (5)

38

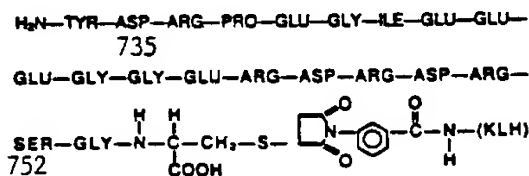
Val	3.60 (4)	3.59 (4)
Ile	0.94 (1)	1.04 (1)
Leu	2.70 (3)	3.12 (3)
Phe	2.00 (2)	2.00 (2)
Lys	2.73 (3)	4.10 (3)
Arg	4.00 (4)	3.90 (4)
$\mu\text{moles/g}$	71	50

<sup>a</sup> Values are uncorrected for destruction during hydrolysis. The number in parenthesis represents the theoretical yield for each amino acid based on the particular sequence.

<sup>b</sup> Not determined.

Briefly, 1 mg of either KLH or BSA in 10 mM sodium phosphate, pH 7.2, was incubated with 4 mg and 800  $\mu\text{g}$  of MBS in dimethylformamide, respectively, for thirty minutes at 25°C. Unreacted MBS and solvent was removed on a Sephadex PD-10 column equilibrated in 50 mM sodium phosphate buffer, pH 6.0. A 100 molar excess of Peptide 4 relative to KLH or BSA, along with approximately 500,000 cpm of  $^{125}\text{I}$  labeled Peptide 4, was added to the reaction mixture and incubated an additional three hours at 25°C. Peptide which was not bound to the protein carrier was removed by repeated dialysis. The coupling efficiency was determined by the amount of  $^{125}\text{I}$  peptide associated with KLH and BSA and was approximately 62% and 56% for KLH and BSA, respectively.

The sequence of the carrier-Peptide 4 conjugate was as follows:





Peptides 3, 5, 8 and 9 (see Table 11) were conjugated to the carrier in the same manner.

Example 12. Induction  
of Immunogenic Response in Rabbits: Peptide-Carrier

Two rabbits were each immunized with 100 µg per dose of Peptide 4-KLH complex, prepared as described above, emulsified in Freund's incomplete adjuvant. The rabbits received one intramuscular injection every two weeks, for a total of three injections, and serum was obtained following each immunization.

A solid phase radioimmunoassay was used to titrate the rabbit anti-peptide antisera. Briefly, 200 ng of Peptide 4 coupled to BSA prepared as described in Example 11 was adsorbed to the wells of polyvinyl microtiter plates, and incubated overnight at 4°C. Following the addition of 10% normal goat serum (NGtS) to block nonspecific sites, the rabbit anti-peptide antisera diluted in 10% NGtS was added and incubated 2 hours at 37°C. Antisera was obtained 14 days after each immunization. The microtiter wells were washed with Tween 20 phosphate buffered saline (T-PBS) and <sup>125</sup>I] goat-anti-rabbit gamma globulin (approximately 500,000 cpm in 50 µl) was added. Following incubation for 1 hour at 37°C, the wells were washed of excess radioactivity with T-PBS, and counted in a gamma counter. All volumes were 50 µl and the anti-peptide titers shown in Table IV are expressed as the reciprocal of the endpoint titer dilution (the highest dilution of antisera that gave cpm above the preimmune rabbit sera). The end point titers were based on fivefold dilutions and represent the mean of triplicate values.

TABLE IV

<u>Rabbit</u>	<u>Immunization</u>	<u>Anti-Peptide 4 Titer</u>
21	Pre-immunization	10
	Primary	1250
	Secondary	6250
	Tertiary	31,250
22	Pre-immunization	10
	Primary	1250
	Secondary	6250
	Tertiary	156,250

The results given in Table IV show that the two rabbits produced a detectible anti-peptide response (as measured by a peptide-BSA) after a single injection of the peptide-KLH. Serum obtained from each rabbit prior to immunization failed to significantly bind the peptide (titers of less than ten). Anti-peptide titers increased following each injection of the peptide and ranged from 31,250 to 156,250 following the third injection.

The specificity of the antibody response was shown by the inability of the anti-peptide sera to bind the control peptide conjugated to BSA. In addition, the HTLV-III peptide 728-745 (Peptide 3) completely inhibited (100%) the binding of the rabbit anti-peptide to peptide-BSA. The two rabbits also produced high antibody titers to KLH; however, rabbit anti-KLH did not bind peptide-BSA.

Example 13. Induction of Immunogenic Response  
in Rabbits: Polyamide Resin-Synthetic Peptide Conjugate

Two rabbits were each immunized with the polyamide resin-peptide 6 conjugate (200 µg of peptide 6 per dose) in Freund's complete adjuvant. Rabbits received an intramuscular injection every two weeks for a total of

three injections. Subsequent injections were given at monthly intervals, and serum was obtained 30 days after the fourth injection. Control anti-peptide antisera was produced in rabbits immunized with either a simian virus 40 tumor antigen peptide coupled to KLH by the method described for the coupling of peptide 4 to KLH in Example 11 or a hepatitis B surface antigen resin bound peptide produced by the method of Example 5.

Example 14. Recognition of HTLV-III Proteins By Rabbit Anti-Peptide Antibodies: Peptides - Carrier

The ability of the rabbit antibodies to Peptide 4 to recognize the native proteins associated with HTLV-III was examined as follows. MOLT-3, an HTLV-III infected T-cell line, was labeled with  $^{35}\text{S}$ -cystine and used for immunoprecipitation as described in Example 2, above, to determine whether the anti-peptide sera would bind any radioactively labeled HTLV-III native proteins. The rabbit anti-peptide antibody from rabbits immunized with the carrier-peptide 4 conjugate prepared as described in Example 11 specifically precipitated a single protein of approximately 160,000 daltons as shown by autoradiographs of SDS-PAGE. This protein is the precursor envelope glycoprotein gp 160 of HTLV-III. No reactivity to HTLV-III proteins was demonstrated when preimmune rabbit sera was used in the immunoprecipitation experiments. The rabbit anti-peptide failed to recognize the gp 120 envelope subunit that is detected with  $^{35}\text{S}$ -cystine labeled MOLT-3 cells when human antisera from AIDS patients is used in immunoprecipitation. The gp 41 envelope subunit does not radioactively label to the same degree with  $^{35}\text{S}$ -cystine as gp 120 and is difficult to detect by immunoprecipitation.

The difficulty of producing gp 41 at a relatively high level of specific radioactivity was circumvented as

follows. HTLV-III infected MOLT-3 cells were double labeled by the addition of both  $^{35}$ [S]-methionine and  $^{35}$ [S]-cystine. The glycoprotein populations present in those double cystine-methionine labeled lysates were then enriched by affinity chromatography on lentil-lectin columns as described in Example 2, above. Both gp 160 and gp 41 glycoproteins was observed when the rabbit anti-peptide sera were reacted with those glycoprotein enriched fractions when analyzed by the radioimmunoprecipitation experiment described in Example 2, above.

Western transfer methods for HTLV proteins verified that the rabbit anti-peptide did recognize gp 41. That method uses stock solutions of infected H9 (BioRad Laboratories, Richmond, Cal.) or MOLT-3 cell lysates as a source of HTLV-III proteins. In that assay,  $5 \times 10^6$  infected cells are solubilized in 1 ml of a 1% Zwittergent 3-14 (Calbiochem-Behring) solution for 5 minutes and centrifugated at  $1000 \times g$  for 10 minutes. The resulting supernatant is mixed with an equal volume of disruption buffer (10 mM Tris-HCl, pH 6.8 glycerol and 0.01% bromphenol blue) and boiled for 3 minutes. Eight  $\mu$ l of disrupted cell lysate is electrophoresed in adjacent lanes in 4-25% linear acrylamide gradient gel (1.5 x 17 x 14 cm) for twenty hours under a constant voltage of 50 V per gel. Electrophoretically separated gradient gels are then transferred to nitrocellulose sheets at 1 amp constant current for 90 minutes at  $10^\circ\text{C}$  using the buffer system described by Towbin, et al., 76 Proc. Natl. Acad. Sci. 4350 (1979), hereby incorporated in its entirety by this specific reference thereto. Pre-stained molecular weight markers (BRL) were also electrophoresed and transferred to nitrocellulose to be used as standards for estimating the molecular weights of the transferred HTLV-III peptides.

After the transfer is completed, the nitrocellulose sheets were incubated with 100 ml of 5% w/v non-fat dry milk rehydrated in PBS containing 0.001% w/v methiolate and 0.0001% v/v Antifoam A (Sigma) for 30 minutes at room temperature. Serial dilutions of sera obtained from the rabbits immunized with Peptide 4 were then incubated with the nitrocellulose sheets for 1 hour at 37°C. Nitrocellulose sheets were then washed with 100 ml of Tween 20 phosphate buffered saline (T-PBS). Biotinylated goat anti-human IgG (5 µg/ml) was then incubated with the nitrocellulose sheets for 1 hour at 37°C in order to detect the binding of the rabbit anti-peptide antibodies. Nitrocellulose sheets were washed again with T-PBS followed by the addition of 1 µg/ml of avidin-labeled horse radish peroxidase (Av-HRP) for 20 min at room temperature. After washing again with T-PBS, 100 ml of a peroxidase chromagen:substrate solution (0.2 mg/ml of O-dianisidine in PBS plus 1 µl/ml of 30% H<sub>2</sub>O<sub>2</sub>) was added to the nitrocellulose membranes until precipitates were observed on the membrane (10-15 min.). The peroxidase catalyzed reaction terminated by washing the nitrocellulose sheets in 2% SDS in water. Controls for the Western transfer assay include the use of normal human sera and a side by side comparison of the reactivity of the antisera with infected and uninfected cell lysates. Binding with the gp 41 protein was observed, as well as with the gp 120 subunit.

Example 15. Recognition of Polyamide Resin-Synthetic Peptides Conjugate by Rabbit Antibodies to Viral Causative Agents of AIDS and ARC

An enzyme-linked immunosorbent assay (ELISA) was used for detection of human antibodies against the viral causative agents of AIDS and ARC. The polyamide resin-peptide 6 conjugate prepared as described in Example 5 was crushed with a mortar and pestle and a

suspension of crushed conjugate was made in borate buffered saline (BBS), pH 8.0. One hundred microliters of that emulsion containing approximately 10  $\mu$ g of peptide 6 (weight basis as calculated by amino acid composition) was absorbed to the solid phase of Dynatech Immulon II microtiter plates in (BBS), pH 8.0, for eight hours at 4°C. Nonspecific sites were blocked with 10% normal goat serum (NGtS) in Tween 20 phosphate buffered saline (T-PBS) and then washed with T-PBS.

Rabbit sera diluted in 10% NGtS was then added to the Peptide 6-coated plates and incubated for one hour at 37°C, followed by washing with T-PBS. Biotin-goat antibody to rabbit IgG (Vector Laboratories, Burlingame, CA) was then incubated with the rabbit sera for one hour at 37°C. The wells were then washed and avidin conjugated to horseradish peroxidase (Av-HRP) was added for 20 minutes at room temperature. The wells were then washed with T-PBS to remove any unbound Av-HRP and peroxidase activity was determined using a .1 mM solution of 1,2'-azino-di(3-ethyl-benzthiazoline-sulfonic acid) (Sigma Chemical Co.) and 0.03%  $H_2O_2$  as substrate. The reaction was stopped with 5% (w/v) sodium dodecyl sulfate in water prior to quantifying spectrophotometrically at 410 nm using a Dynatech plate reader. Optimal dilutions of each reagent were selected by titration. All reagents for determining specific binding except the substrate were diluted in 10% NGtS. The resin-bound hepatitis B surface antigen described in Example 13 served as a control. The results are shown in Fig. 4, in which the results for the polyamide resin-peptide 6 conjugate are shown in graph A and the results for the polyamide resin-hepatitis B peptide conjugate are shown in graph B. Rabbit anti-peptide antisera were obtained from: rabbit no. 1 (●); rabbit

no. 2 (o). All tests were performed in triplicate and the brackets refer to the range of values.

Example 16. Neutralization of Viral Causative  
Agents of AIDS and ARC By Antibodies to Polyamide  
Resin-Peptide 6 Conjugate

A. HTLV-III Replication

Prior to examining the ability of the rabbit anti-polyamide resin-peptide 6 antisera to neutralize HTLV-III infectivity in vitro, it was necessary to determine the kinetics of HTLV-III replication in a susceptible human T-cell line. Various dilutions of an HTLV-III viral stock were incubated with normal human and preimmune rabbit serum similar to the methods utilized to examine viral neutralization with the anti-peptide reagents. Following a 1 hour incubation with the sera, the HTLV-III viral dilutions were added to cultures of susceptible A3.01 cells maintained according to the methods set out by Folks, T. et al. (1985 Proc. Nat'l Acad. Sci. USA 4539-4543 (1985)), hereby incorporated in its entirety by this specific reference thereto. Cultures infected with HTLV-III were maintained for 15 days and supernatant fluid from the cultures was removed on days 5, 8, 10, 12 and 15. HTLV-III replication was assessed by the presence of reverse transcriptase (RT) activity in the culture supernatant fluids.

The methods for performing reverse transcriptase activity are described in detail elsewhere (Barre-Sinoussi, F., et al., 220 Science 868-871 (1983). Briefly, 15  $\mu$ l of each supernatant from A3.01 infected cultures were added to 96 well microtiter plates that contain 50  $\mu$ l of virus dilution buffer (0.05 M Tris-HCl, pH 7.8, 0.1 M NaCl, 0.15 mg/ml dithiothreitol (DTT), 0.1% Triton X-100). Fifty  $\mu$ l of the reaction mixture (1 M Tris-HCl, 3 M KCl, 0.15  $MgCl_2$ , 10% Triton, poly A,

oligo DT and  $^3\text{H}$ -thymidine tri-phosphate ( $^3\text{H}$ -TTP) was added and incubated 1 hour at  $37^\circ\text{C}$ . Following the incubation, 50  $\mu\text{l}$  of the mixture was dotted on nitrocellulose filter papers. The filter papers were washed successively in beakers containing: (1) 5% trichloroacetic acid (TCA) and 5% sodium pyrophosphate; (2) 5% TCA; and (3) 50% ethanol. The filter papers were counted in an automatic scintillation counter and the cpm of  $^3\text{H}$ -TTP was determined.

To prepare the various dilutions of the HTLV-III viral stock, one normal human serum and the 4 preimmune rabbit sera was heat inactivated at  $56^\circ\text{C}$  for 1 hour and filter sterilized through a  $0.2\ \mu\text{m}$  filter. One hundred fifty microliters of a 1:5 dilution was incubated with an equal volume of  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$  and  $10^{-5}$  dilutions of an HTLV-III isolate, termed NY-5 for 1 hour at  $37^\circ\text{C}$ . The NY-5 isolate has an infectious titer of  $10^{-5}$  units as determined on the human T-cell line A3.01. Following the incubation, the antibody treated virus mixture was added to  $10^6$  A3.01 cells. The mixture was incubated for 2 hours at  $37^\circ\text{C}$  in the presence of 1  $\mu\text{g}/\text{ml}$  of POLYBRENE (Cal Biochem). The infected A3.01 cells were washed and resuspended in 1 ml of RPMI media containing 10% heat inactivated fetal calf serum and dispersed into 24 well microtiter plates. Five hundred microliters of spent media supernatant was removed at days 5, 8, 10, 12 and 15 after infection and frozen at  $-135^\circ\text{C}$  until reverse transcriptase activity was determined. Following the removal of supernatant, the individual cultures were fed with 500  $\mu\text{l}$  of RPMI plus fetal calf serum. Each culture is performed in duplicate. RT activity was determined by the counts per minute of  $^3\text{H}$ -TTP incorporated.

Pooled human AIDS serum that tests positive by ELISA and Western blot were obtained from Dr. Thomas



Folks, Laboratory of Immunoregulation, NIAID, Bethesda, MD. The human sera and rabbit anti-peptide antisera were treated as described above. In each instance, the preimmune sera of that particular rabbit served as the negative control indicative of no neutralization of HTLV-III infectivity as determined by RT activity cpm when compared to the individual rabbit anti-peptide preparation. The preimmune and rabbit anti-peptide antisera were incubated with  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ , and  $10^{-5}$  dilutions of the NY-5 isolate as described above. Each culture was performed in duplicate. Supernatants from infected A3.01 cells were removed at days 5, 8, 10, 12 and 15, frozen at  $-135^{\circ}\text{C}$ , and assayed for RT activity. The percent inhibition of RT activity was determined by the following formula:

$$1 - \frac{\text{cpm RT assay of anti-peptide antisera cultures}}{\text{cpm RT assay of preimmune sera cultures}} \times 100$$

Background counts ranging from 200 to 750 cpm were subtracted from each determination prior to calculating percent inhibition.

The kinetics of four dilutions of HTLV-III (A,  $10^{-1}$ ; B,  $10^{-2}$ ; C,  $10^{-3}$ ; D,  $10^{-4}$ ) based on RT activity at days 5, 8, 10, 12 and 15 following infection are shown in Figure 5. The points on the curve reflect the mean cpm of  $^3\text{H-TTP}$  uptake based on five different determinations performed in duplicate. The range bars signify the standard error of the mean. Significant replication of HTLV-III diluted  $10^{-1}$  and  $10^{-2}$  did not occur until 10 days after infection. At 12 days the virus was also actively replicating and by 15 days, the decrease in RT activity indicated the cytolytic action of these dilutions of HTLV-III for the susceptible target cells. Replication of the  $10^{-3}$  dilution of HTLV-III was observed at days 12 and 15, whereas little or no replication was demonstrated with  $10^{-4}$  dilution of

virus even by day 15. Readings of greater than 2000 cpm of  $^3\text{H}$ -uptake were selected as an indication of HTLV-III replication based on the fact that 11 out of 12 determinations (days 5 and 8 with all dilutions of virus, day 10 for  $10^{-3}$  and  $10^{-4}$  dilutions and days 12 and 15 for  $10^{-4}$  dilution) with less than 2000 cpm had standard deviations and standard errors of the mean greater than or equal to the mean cpm.

The finding that the standard deviations and standard errors of the mean equalled or exceeded the mean cpm at those dilutions indicated that the individual cpm values were extremely variable and that a determination of whether low RT activity resulted from specific neutralization or just random variation of the RT assay would be difficult. Antibodies may not efficiently neutralize virus if overwhelming quantities of infectious virions are present. Based on the kinetic studies and the nature of the HTLV-III viral stock, it was determined that neutralization of HTLV-III infectivity by human and rabbit anti-peptide antisera would be examined at days 10, 12, and 15 for the  $10^{-1}$  and  $10^{-2}$  dilution and days 12 and 15 for the  $10^{-3}$  dilution of HTLV-III.

#### B. Neutralization of Anti-Peptide Antisera

As indicated by the results shown in Table V, the antiserum to the polyamide resin-peptide 6 conjugate from one rabbit efficiently reduced HTLV-III replication at day 10 when compared to pooled human sera from AIDS patients at both  $10^{-1}$  and  $10^{-2}$  dilutions of virus. A second rabbit anti-serum to that peptide failed to reduce HTLV-III replication and served as a control antiserum throughout the RT assay. No anti-HTLV-III activity was detected in this particular antiserum based on radioimmunoprecipitation even though the rabbit received a similar immunogen and produced a detectable

anti-peptide response. The antiserum that neutralized HTLV-III detected both the gp 120 and gp 160 envelope glycoproteins. Rabbit no. 1 antiserum was found to be less efficient in neutralizing HTLV-III when compared to human AIDS serum on day 12 and 15. The percent reduction of RT activity decreased by day 12 from greater than 90 percent (day 10) to 23 and 45 percent for a  $10^{-1}$  and  $10^{-2}$  dilution of virus, respectively. The more dilute the virus, the greater the reduction of RT activity at day 12, indicating that the ability of the antisera to neutralize is dependent on the amount of virus.

Both rabbit antisera to the polyamide resin-peptide 6 conjugate neutralized a  $10^{-2}$  virus dilution at day 10 but neither were as efficient at neutralizing higher concentrations of virus ( $10^{-1}$ ) when compared with the serum from a human AIDS patient or the rabbit anti-polyamide resin-peptide 6 conjugate. Rabbit no. 3 neutralized efficiently (greater than 95%) a  $10^{-3}$  virus dilution at both 12 and 15 days. No reduction in RT activity was obtained with antiserum from rabbit no. 4 on day 15, which may be reflected in the fact that rabbit no. 3 had a higher antibody titer to HTLV-III when compared to rabbit no. 4. No reduction of RT activity was observed on day 15 with any of the antisera at the high concentrations of virus indicating that infectious virus was present in the culture and the antisera were not effective in inhibiting viral replication at that point in time. Both antisera from the human AIDS patient and rabbit no. 3 inhibited RT activity at day 15 with a  $10^{-3}$  dilution of virus. Control rabbit antisera produced against non-HTLV-III envelope glycoproteins coupled to KLH and the hepatitis B surface antigen control resin bound peptide preparation at similar dilutions employed in the in

vitro neutralization test demonstrated no significant inhibition of RT activity (less than 25%).

TABLE V

Virus Dilution	Human AIDS Serum	Rabbits			
		Anti-503-532 peptide	Anti-735-752 peptide	Anti-735-752 peptide	Anti-735-752 peptide
		1	2	3	4
Percent reduction of RT activity (10 days)					
10 <sup>-1</sup>	98%	96%	0%	0%	0%
10 <sup>-2</sup>	97%	94%	0%	90%	97%
10 <sup>-3</sup>	nd	nd	nd	nd	nd
Percent reduction of RT activity (12 days)					
10 <sup>-1</sup>	91%	23%	0%	0%	0%
10 <sup>-2</sup>	97%	45%	0%	67%	67%
10 <sup>-3</sup>	100%	70%	0%	100%	nd <sup>a</sup>
Percent reduction of RT activity (15 days)					
10 <sup>-1</sup>	0%	0%	0%	0%	0%
10 <sup>-2</sup>	0%	0%	0%	0%	0%
10 <sup>-3</sup>	90%	24%	0%	95%	0%

<sup>a</sup> The number of cpm of <sup>3</sup>H uptake in that particular culture was less than 2000 cpm and the percent reduction of RT activity was not determined.

Example 17. Assay for Diagnosis  
of AIDS or ARC: Detection of Antibodies

An insoluble support matrix is coated with 5 µg each of the polyamide resin-synthetic peptide conjugates

prepared as described above in Example 5 in borate buffer saline (BBS), pH 8.0, for 8 hours at 4°C. (Alternatively, the matrix may be coated for one hour at 37°C). The conjugate is blocked for 20 minutes with 10% normal goat serum (NGtS), and washed three times with Tween 20 phosphate buffered saline (T-PBS). A serum sample suspected of containing antibody to the viral causative agents of AIDS and/or ARC is added and incubated for one hour at 37°C. The support matrix is washed three times with T-PBS, and biotin-labeled goat anti-human Ig (1:1000 of 5 mg/ml in 10% NGtS, Vector Labs, Burlingame, California) is added. The matrix is washed three times with T-PBS, and a 1:2000 of 5 mg/ml avidin-horseradish peroxidase is added and incubated for twenty minutes at room temperature. The matrix is washed three times with T-PBS and the substrate, the diammonium salt of 2, 2'-azinodi-(3-ethylbenzthiazoline sulfonic acid) (ABTS) with  $H_2O_2$ , is added. The enzyme reaction is stopped with 10% SDS and optical density is read at 410 nm as described in Example 15.

Example 18. Assay for Diagnosis of AIDS or ARC:  
Detection of Antigen

To detect the presence of the AIDS antigen, the solid phase matrix is coated with antibodies produced by immunization of an experimental animal with one or more of the polyamide resin-peptide conjugates prepared as described in Example 5, and the antibodies blocked and washed as described above. The biological fluid sample suspected of containing the AIDS or ARC viral causative agent is then added and washed. The assay can be conducted either as a direct binding assay or as an inhibition assay. If a direct binding assay is conducted, biotin-labeled antibodies to the AIDS and/or ARC virus produced as described above are added and washed. The avidin-labeled enzyme is then added as

described above and washed, and the substrate is added as described above. The reaction is stopped and the optical density is read.

If conducted as an inhibition assay, instead of adding biotin-labeled antibody to AIDS virus, the biotin-labeled synthetic peptide is added and the insoluble support matrix is washed. The avidin-labeled enzyme is then added and washed. The substrate is added, the reaction stopped and optical density is read. The IgG from human or chimpanzee AIDS-containing serum is purified by ion exchange chromatography on a Whatman DE-52 anion exchange column. IgG from rabbit anti-peptide is purified with a protein A-Sepharose 4B column (Pharmacia). The IgG is biotinylated using biotin-N-hydroxysuccinamide ester (Boehringer Mannheim).

Example 19.

Vaccination Against AIDS and ARC

To vaccinate an experimental animal against the viral causative agents of AIDS and ARC, the synthetic peptides 1-9 are synthesized on the polyamide resin as described in Example 5, above. The polyamide resin-synthetic peptide conjugate, or a mixture of several conjugates, is injected into the animal in a bolus of between 100 to 1000 µg of synthetic peptide in alum as an adjuvant. Three separate injections may be given, either intramuscularly or subcutaneously, on a biweekly basis until a measurable antibody response to the virus is detected. Other time intervals such as 0, 1 and 6 months may also be used for the injection of the synthetic peptide.

Example 20.

Screening of Putative AIDS Vaccines

The synthetic peptide of the present invention can also be used to screen potential AIDS vaccine candidates

for their ability to induce an immunogenic response in an animal subject. One or more of the polyamide resin-synthetic peptide conjugates are coated onto the insoluble matrix as described above in Example 15. The vaccine candidate is then incubated with antibodies against the peptide (with or without biotin labelling). If biotin labeled, the avidin-enzyme is added, if not, a biotin anti-species antibody such as biotin goat anti-rabbit IgG is added, followed by the addition of the avidin-enzyme. The substrate is added, the reaction stopped and optical density read to determine the ability of the vaccine candidate to block the binding of the peptide.

\* \* \* \* \*

The preceding examples are presented by way of exemplification only and not by limitation. Variations in these methods will be known to those skilled in the art, and it is expected that all such variations will be made without departing from the spirit and scope of the present invention as claimed in the following claims.

## WHAT IS CLAIMED IS:

1. A composition of matter capable of inducing an immunogenic response to the viral causative agents of AIDS and ARC comprising a polyamide resin and a synthetic peptide comprising a chain of amino acids having a sequence homologous to a portion of the amino acid sequence of the gp 120 or gp 41 envelope glycoprotein of HTLV-III, ARV or LAV and having a hydrophilic region therein.

2. The composition of claim 1 wherein said chain of amino acids includes a  $\beta$  turn.

3. The composition of claim 1 wherein said polyamide resin comprises a cross-linked polydimethylacrylamide resin.

4. The composition of claim 1 wherein said chain of amino acids is conjugated to said polyamide resin through a linker.

5. The composition of claim 4 wherein said linker is an oxyalkyl benzoic acid derivative.

6. A method of immunizing an experimental animal against the viral causative agents of AIDS and ARC comprising synthesizing a peptide comprising a chain of amino acids having a sequence homologous to a portion of the gp 120 or gp 41 envelope glycoproteins of HTLV-III, ARV or LAV and having a hydrophilic region therein on a polyamide resin and administering an immunogenically effective amount of the polyamide resin-peptide conjugate to an experimental animal.

7. The method of claim 6 wherein said polyamide resin-peptide conjugate is administered to said animal in a pharmaceutically acceptable diluent.

8. The method of claim 7 wherein said diluent additionally comprises an adjuvant.

9. The method of claim 7 wherein said diluent is either distilled water or a neutral pH buffer.



10. A method of detecting antibodies against the viral causative agents of AIDS and ARC comprising:

conjugating a polyamide resin-synthetic peptide conjugate to the ligand of a specific binding pair wherein said binding pair is comprised of said ligand and an anti-ligand having specific affinity for said ligand and said synthetic peptide is comprised of a chain of amino acids having a sequence homologous to a portion of the gp 120 or gp 41 envelope glycoproteins of HTLV-III, ARV or LAV;

contacting said conjugate with sera from an animal, thereby causing any antibodies to the viral causative agents of AIDS or ARC in said sera to bind to said conjugate; and

thereafter contacting the bound antibodies with the anti-ligand of said specific binding pair.

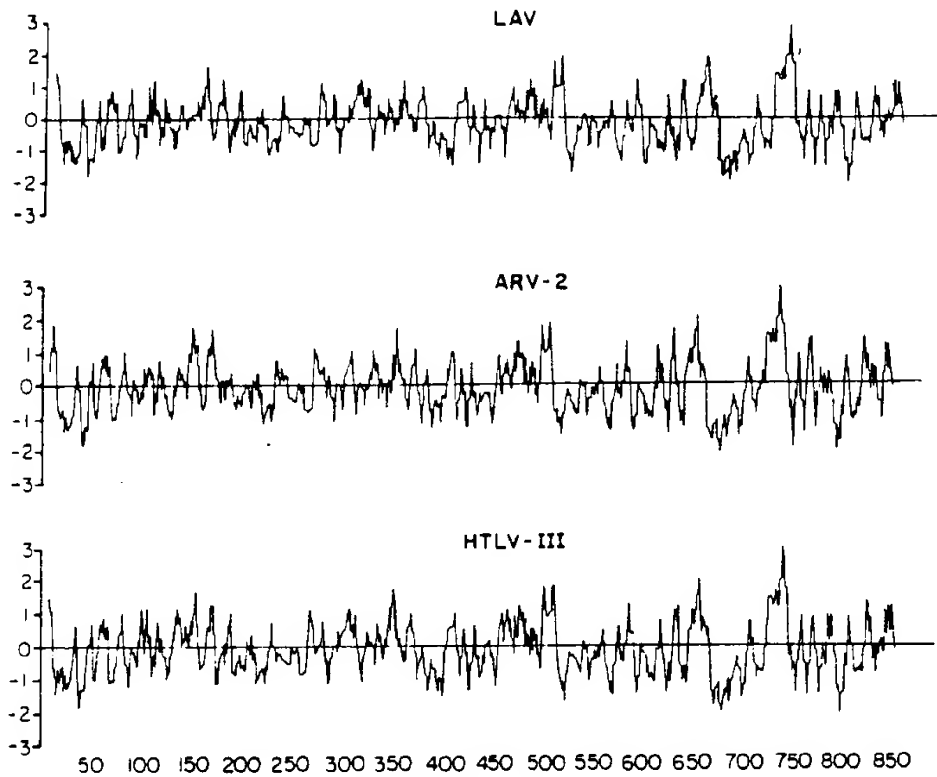
11. The method of claim 10 wherein the ligand is an enzyme and the anti-ligand is a substrate for which said enzyme is specific.

12. The method of claim 11 wherein the enzyme is horseradish peroxidase and the substrate is hydrogen peroxide.

13. The method of claim 10 wherein the ligand is an antibody and the anti-ligand is an antigen for which the antibody is specific.

14. The method of claim 10 wherein the ligand is an antigen and the anti-ligand is an antibody specific for said antigen.

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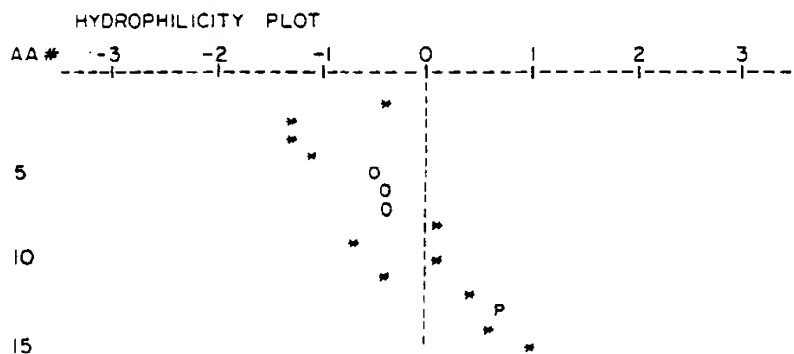
**FIG. 1**

2/4

AA #	AA CODE	H VALUE	H AVERAGE
1	K--LYS	3	-.3
2	E--GLU	3	-1.183
3	Y--TYR	-2.3	-1.183
4	A--ALA	-.5	-1.1
5	F--PHE	-2.5	-.517
6	F--PHE	-2.5	-.4
7	Y--TYR	-2.3	-.283
8	K--LYS	3	.1
9	L--LEU	-1.8	-.7
10	D--ASP	3	.1
11	I--ILE	-1.8	-.367
12	I--ILE	-1.8	.433
13	P--PRO	0	.667
14	I--ILE	-1.8	.6
15	D--ASP	3	.95
16	N--ASN	.2	
17	D--ASP	3	
18	T--THR	-.4	
19	T--THR	-.4	
20	S--SER	.3	

PEAK VALUES ARE:

PEAK #	AA POS.	AVE VALUE
1	15	.95
2	8	.1

**FIG. 2**

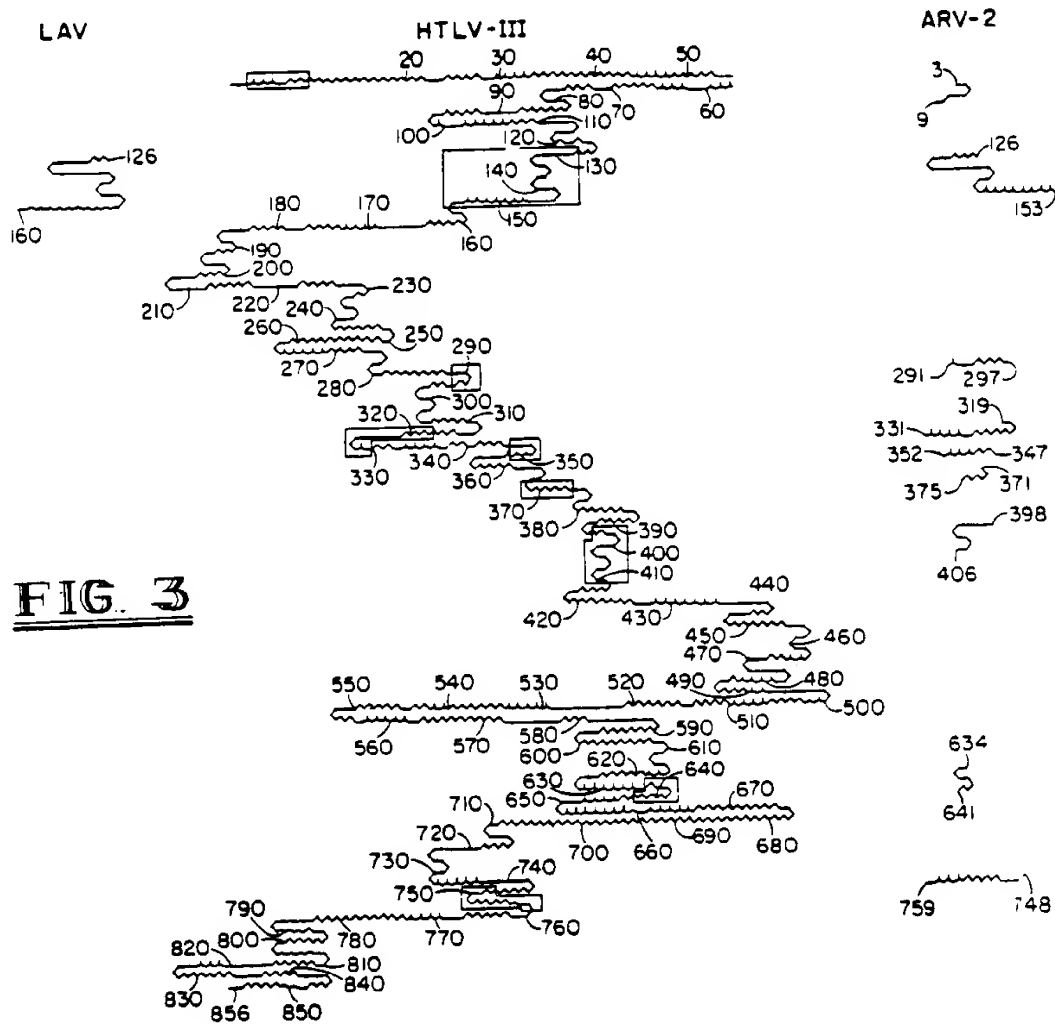


FIG. 4

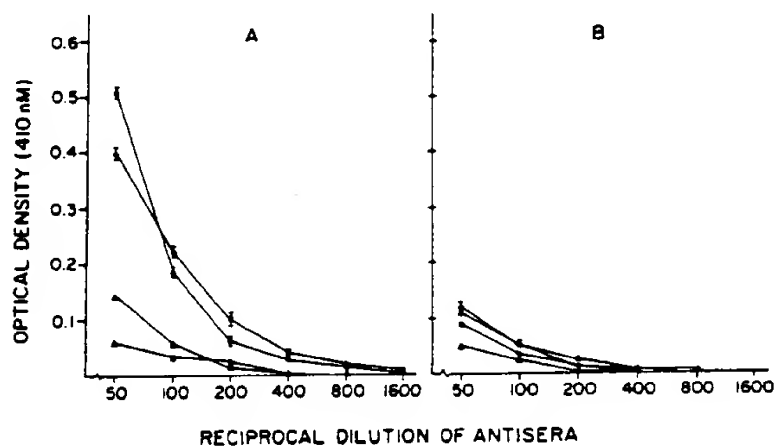
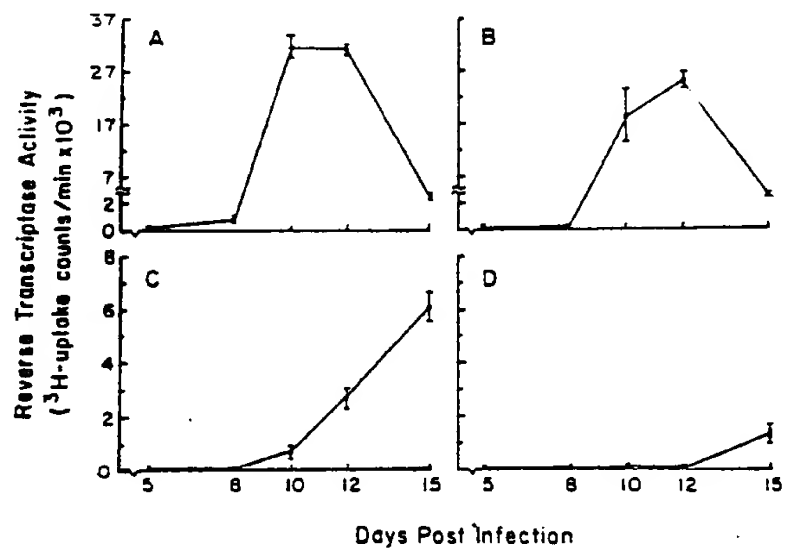


FIG. 5



# INTERNATIONAL SEARCH REPORT

International Application No PCT/US87/01733

## I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) <sup>1</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): A61K 39/12; C12Q 1/70; C07K 7/10, 17/08; G01N 33/53  
U.S.C1.: 424/88, 89; 435/5, 7, 28; 530/324, 334, 403, 405, 815

## II. FIELDS SEARCHED

Minimum Documentation Searched <sup>4</sup>

Classification System

Classification Symbols

U.S. 424/88, 89  
435/5, 7, 28  
530/324, 334, 403, 405, 815

Documentation Searched other than Minimum Documentation  
to the extent that such Documents are included in the Fields Searched <sup>5</sup>

## III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>14</sup>

Category <sup>6</sup> Citation of Document, <sup>15</sup> with indication, where appropriate, of the relevant passages <sup>17</sup> Relevant to Claim No. <sup>18</sup>

- |      |   |      |
|------|---|------|
| X    | Analytical BioChemistry, Volume 151, no. 2, issued 1985 December (New York), D. PAULETTI et al, "Application of a Modified Computer Algorithm in Determining Antigenic Determinants Associated with the AIDS Virus Glycoprotein" pages 540-546, (see pages 541-542 and 544-545)". | 1-14 |
| Y    | Nature, Volume 313, issued 1985, February 7 (London) M.A. MUESING et al, "Nucleic Acid Structure and Expression of the Human AIDS/lymphadenopathy Retrovirus" pages 450-458 (see page 455).   | 1-14 |
| X, P | Science, Volume 231, issued 1986, March 8 (Washington, D.C.) R.C. KENNEDY et al, "Antiserum to a Synthetic Peptide Recognizes the HTLV-III Envelope Glycoprotein", pages 1556-1559, (see page 1556).  | 1-9  |

\* Special categories of cited documents: <sup>19</sup>

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Z" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search <sup>1</sup>

21 SEPTEMBER 1987

International Searching Authority <sup>1</sup>

ISA/US

Date of Mailing of this International Search Report <sup>2</sup>

14 OCT 1987

Signature of Authorized Officer <sup>10</sup>

Christine M. Nucker

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages 1:	Relevant to Claim No 1:
Y	Bio/Technology, Volume 3, issued 1985 October (New York) T.W. CHANG et al, "Detection of Antibodies to Human T-Cell Lymphotropic Virus-III (HTLV-III) with an Immunoassay Employing a Recombinant Escherichia coli-Derived Viral Antigenic Peptide" pages 905-909 (see page 905).	10-14
A	Science, Volume 228, issued 1985 May 31 (Washington, D.C.) J.S. ALLAN et al, "Major Glycoprotein Antigens that Induce Antibodies in AIDS Patients are Encoded by HTLV-III" pages 1091-1094.	
Y,P	Proceedings of The National Academy of Sciences USA, Volume 83, issued 1986 September (Washington, D.C.) W.G. ROBEY et al, "Prospect for Prevention of Human Immunodeficiency Virus Infection: Purified 120-KDa Envelope Glycoprotein Induces Neutralizing Antibody" pages 7023-7027.	1-9
Y,P	Proceedings of The National Academy of Sciences USA, Volume 83, No. 24, issued 1986 December (Washington, D.C.) T.J. MATTHEWS et al, "Restricted Neutralization of Divergent Human T-lymphotropic Virus Type III isolates by Antibodies to the Major Envelope Glycoprotein" pages 9709-9713 (see page 9709).	1-9
X	Science, volume 233, issued 1986 July 11 (Washington, D.C.) L.A. LASKY et al, "Neutralization of the AIDS Retrovirus by Antibodies to a Recombinant Envelope Glycoprotein" pages 209-212 (see page 209).	1-9
Y,P	Nature, Volume 323, issued 1986 October 23 (London) J.D. LIPSON et al, "Induction of CD4-dependent cell fusion by the HTLV III/LAV envelope glycoprotein" pages 725-728 (see page 726).	1-9
Y,P	US, A, 4,629,783 (W.L. COSAND) 16 December 1986 (16.12.86), see column 7, lines 30-56; column 9, lines 37-49.	1-14
Y	US, A, 4,474,757 (R. ARNON et al) 2 October 1984 (02.10.84) see abstract.	1-9

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages <sup>1</sup>	Relevant to Claim No. <sup>1</sup>
A	Cancer Research, Volume 45 (Suppl.) issued 1985 September (Baltimore, Maryland USA) M.G. SARNGADHARAN et al, "Immunological Properties of HTLV-III Antigens Recognized by Sera of Patients with AIDS-related Complex and of Asymptomatic Carriers of HTLV-III Infection" pages 4574-4577s.	
A,P	Proceedings of The National Academy of Sciences USA, Volume 83, issued 1986 August (Washington, D.C.) J.J.G. WANG et al, "Detection of Antibodies to Human T-lymphotropic Virus Type III by using a Synthetic Peptide of 21 Amino Acid Residues Corresponding to a Highly Antigenic Segment of gp 41 envelope protein" pages 6159-6163.	
A,P	Journal of General Virology, Volume 67, issued 1986 (Great Britain) J. SCHNEIDER et al, "Shedding and Interspecies Type Sero-reactivity of the Envelope Glycoprotein gp120 of the Human Immunodeficiency Virus" pages 2533-2538.	
Y	Cell, Volume 41, issued 1985 July (Cambridge, Massachusetts, USA), R. CROWL et al, "HTLV-III envGene Products Synthesized in E. coli are Recognized by Antibodies Present in The Sera of AIDS Patients" pages 979-986 (see pages 981-982).	1-10
A	Science, Volume 229, issued 1985 September 27 (Washington, D.C.) F. VERONESE et al, "Characterization of gp41 as the Transmembrane Protein Coded by the HTLV-III/LAV Envelope Gene" pages 1402-1405.	



## FURTHER INFORMATION: CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE <sup>10</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . . . because they relate to subject matter <sup>12</sup> not required to be searched by this Authority, namely:

2. ☐ Claim numbers . . . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out <sup>13</sup>, specifically:

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING <sup>14</sup>

This International Searching Authority found multiple inventions in this international application as follows:

I. Claims 1-9 drawn to a polyamide resin conjugated synthetic peptide and method of immunizing using same; Class 424/88 or 514/2.

II. Claims 10-14 drawn to methods of detecting antibodies; Class 436/528.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. Telephone Practice

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Attachment to Form PCT/ISA/210 Part VI. 1

PCT/US87/01733

Telephone Approval:

\$140 payment as approved by Donald R. Comuzzi on 18 September 1987 for Group II; charge to deposit account no. 03-3483.

Counsel was advised that he has no right to protest for any group not paid for and that any protest must be filed no later than 15 days from the date of the mailing of the search report (Form 210).

Reasons for holding lack of unity of invention:

The invention as defined in Group I (claims 1-9) drawn to a polyamide resin conjugated synthetic peptide and method of immunizing using same classified in class 424, subclass 88 or class 514, subclass 2 may be used in materially different processes than the invention of group II (claims 10-14) drawn to methods of detecting antibodies, classified in class 436, subclass 528; and have different protocol, as one involves in vivo processes and one involves in vitro processes.

Time Limit for Filing a Protest

Applicants are hereby given 15 days from the mailing date of this Search Report in which to file a protest of the holding of lack of unity of invention. In accordance with PCT Rule 40.2 applicants may protest a holding of lack of unity only with respect to the group(s) paid for.